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		(54) Title: IMMUNOSTIMULATORY COMPOSITION AND METHOD (57) Abstract Disclosed are therapeutic compositions and methods for inducing cytotoxic T cell responses <i>in vitro</i> and <i>in vivo</i> . The therapeutic compositions consist of antigen presenting cells activated by contact with a polypeptide complex constructed by joining together a dendritic cell-binding protein and a polypeptide antigen. Also disclosed are expression vectors and systems for producing the polypeptide complexes.

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IMMUNOSTIMULATORY COMPOSITION AND METHOD

5 Field of the Invention

The present invention relates to compositions and methods for stimulation of specific cellular immune responses *in vivo*. More specifically, the invention is concerned with elimination of tumor cells by cytotoxic T-lymphocytes (CTL) activated *in vivo* or *in vitro* by exposure to antigen presenting cells exposed to an immunostimulatory polypeptide complex.

10

Background of the Invention

The immune response of the mammalian immune system is divided into two general types: humoral immunity, mediated largely by circulating antibodies, and cellular immunity mediated by various forms of T-cells. Extracellular antigens stimulate a humoral response, while
15 intracellular antigens such as viruses, stimulate a cellular response.

The cellular immune response to virally infected cells and tumor cells is largely mediated by cytotoxic T-lymphocytes (T_c, or CTL), when they recognize foreign antigens attached to the host cell surface as part of the Major Histocompatibility Complex (MHC), and more particularly, a common form of MHC known as MHC Class I. In contrast, antigens derived
20 from non-viral pathogens (bacteria, fungi) are generally expressed as part of an MHC Class II complex. A different subpopulation of effector T cells (cell mediated immune cells; CMI) release cytokines that activate the host cell to destroy such pathogens.

In experimental systems, tumor-antigen specific CTL are the most powerful immunological mechanism for the elimination of tumors. CTL can be induced either *in vivo* with vaccines or
25 can be generated *in vitro* and then be re-infused into the tumor-bearing organism. The *in vivo* induction of CTL is typically accomplished by immunization with live virus or cells (Tanaka, et al., J.Immunol., (1991), 147, 3646-52, Wang, et al., J.Immunol., (1995), 4685-4692, Torre-Amione, et al., Proc.Natl.Acad.Sci.U.S.A., (1990), 87, 1486-90).

Except for a few special viral proteins such as the SV-40 large T-antigen and the Hepatitis
30 B surface antigen, injection of isolated or soluble proteins does not result in induction of CTL (Schirmbeck, et al., Eur.J.Immunol., (1993), 23, 1528-34). CTL are induced when a protein enters the class I pathway of antigen processing. To enter this pathway the protein must be present in the cytosol of the APC. There it is degraded into peptides which are then transported into the endoplasmic reticulum, where they associate with nascent HLA class I
35 molecules. These peptides are then displayed together with the class I molecules on the cell surface and can serve as an inducer and target of class I restricted antigen-specific CTL. Physiologically, only proteins that are endogenously synthesized by an APC enter this pathway.

Non-cellular-delivery vehicles for proteins, such as pH-sensitive liposomes, can overcome the requirement for endogenous synthesis *in vivo* (Nair, et al., J.Exp.Med., (1992), 175, 609-12, Nair, et al., J.Virol. (1993), 67, 4062-9); however, these treatments are also quite toxic to the target cells.

5 Induction of primary HLA class I restricted CTL by pure soluble proteins *in vitro* has not been reported. The most common tool for *ex vivo* induction of primary CTL are small (8-11-mer) synthetic peptides (Staass, et al., Proc.Natl.Acad.Sci.U.S.A. (1992), 89, 7871-5, Carbone, et al., J.Exp.Med. (1988), 167, 1767-79). These synthetic peptides associate with class I molecules on the cell surface without the requirement for endogenous processing. When
10 presented on the surface of an appropriate APC (such as a dendritic cell) they can then induce a primary CTL response. However, frequently these CTL do not protect against challenge with pathogens that endogenously synthesize the protein from which the peptide was derived because of their low T-cell receptor avidity (Speiser, et al., J.Immunol. (1992), 149, 972-80) and because they induce reactivity with a single epitope of the target antigen.

15 GM-CSF is a cytokine that has pleiotropic function both in hematopoiesis as well as in immunology. GM-CSF has been shown to promote differentiation and survival of dendritic cells. GM-CSF can be used as an systemic adjuvant (Jones, et al., Eur.J.Clin.Microbiol.Infect.Dis. (1994), 13, S47-53).

It is well known that immunization with soluble proteins can result in a significant antibody
20 response. However, since class II restricted antigen presentation or direct B-cell stimulation is responsible for this effect, antibody induction has no predictive value for the stimulation of class I mediated induction of CTL. Most proteins that induce antibodies *in vivo* fail to induce CTL.

GM-CSF fusion proteins have been shown to induce *in vivo* antibody responses in a
25 lymphoma mouse model (Levy, R. and Tao, M.-H. (1993) Nature 362: 755-758; Chen, et al., J.Immunol. (1995), 3105-3117). In this study, tumor idiotype fused to GM-CSF was found to be superior to the mixture of both molecules and to other conventional adjuvants for the induction of antibody responses. In contrast to other solid tumors, antibody responses are believed to be the effector mechanism for tumor protection and for tumor therapy in
30 lymphoma.

Moreover, *in vitro* induction of immunity is generally much more difficult to achieve for both cellular and humoral responses. For example, viral antigen-transfected fibroblasts induce Class I restricted CTL *in vivo* in mice but fail to do so *in vitro* (Kündig, et al., Science (1995), 268, 1343-1347). Therefore, an antibody induction study with GM-CSF fusion proteins *in vivo*

does not imply any of its *in vitro* utilities, and is particularly unproductive of CTL induction *in vitro* or *in vivo*.

Other methods that have been used for *in vitro* induction of primary protein-derived CTL are osmotic shock of dendritic cells and the use of pH-sensitive liposomes (Nair, et al., J. Exp. Med. (1992), 175, 609-12). However, such methods have been shown to be inherently ineffective and toxic to APC's, because they disrupt cellular membranes by physical and chemical force in order to release the protein antigen into the cytoplasm.

These limitations are overcome by the discovery encompassed by the present invention. It is the discovery of the present invention that a T-cell response, and specifically, an MHC-Class I mediated T-cell response, can be stimulated by an isolated or soluble protein, when it is presented to the immune system as part of a complex with a dendritic cell binding protein, and more particularly GM-CSF. It is the further discovery of the present invention that such a response can be stimulated *in vitro*. As discussed above, *in vitro* stimulation of such a response has not previously been demonstrated using a full-length soluble antigen. The present invention provides for induction by isolated or soluble proteins of cellular immunity *in vitro* by presenting a specific antigen to an antigen presenting cell (APC), such as a dendritic cell, as part of an immunogenic fusion protein.

An important aspect of the present invention is the choice of fusion partner protein: a dendritic cell binding protein, such as granulocyte-macrophage colony stimulating protein (GM-CSF). Without relying on any particular mechanistic theory, it is believed that the protein antigen is transported over the plasma membrane of the APC in a receptor-mediated, non-disruptive fashion. It is further believed that the dendritic cell binding portion of the fusion protein serves to preserve the viability and functionality of the APC.

An additional aspect of the invention relates to the choice of target antigen. Although several tumor related antigens have been shown to serve as targets for T-cell mediated immunity *in vivo*, *in vitro* induction by isolated soluble polypeptide antigens has not been demonstrated. (Fisk, et al., J. Exp. Med. (1995), 181, 2109-2117). In experiments carried out in support of the present invention, it has now been demonstrated that tumor associated proteins not previously shown to be target antigens for CTL can become such targets by priming CTL with GM-CSF fusion derivatives *in vitro*.

Summary of the Invention

In one aspect, the invention is directed to a therapeutic composition for stimulating a cellular immune response. The composition is an isolated, stimulated potent antigen presenting

cell, such as an activated dendritic cell, that is able to activate T-cells to produce a multivalent cellular immune response against a selected antigen. In general, the measured T-cell response is substantially higher than a T-cell response produced by such potent antigen presenting cells stimulated by the selected antigen alone. In a preferred embodiment of the invention, the
5 potent antigen presenting cell is stimulated by exposing the cell *in vitro* to a polypeptide complex that consists essentially of a dendritic cell-binding protein and a polypeptide antigen. Preferably, the polypeptide antigen is either a tissue-specific tumor antigen or an oncogene gene product. However, it is appreciated that other antigens, such as viral antigens can be used in such combination to produce immunostimulatory responses.

10 In another preferred embodiment, the dendritic cell-binding protein that forms part of the immunostimulatory polypeptide complex is GM-CSF. In a further preferred embodiment, the polypeptide antigen that forms part of the complex is the tumor-specific antigen prostatic acid phosphatase. In still further preferred embodiments, the polypeptide antigen may be any one of the oncogene product peptide antigens Her2, p21RAS, and p53. The polypeptide complex
15 may also contain, between the dendritic cell-binding protein and the polypeptide antigen, a linker peptide.

In a related aspect, the invention includes a method of activating an isolated antigen presenting cell *in vitro*. According to the method, the activation includes contacting an isolated antigen presenting cell with a polypeptide complex. The polypeptide complex used in this
20 method is as described above; that is, it consists essentially of a dendritic cell-binding protein covalently linked to a polypeptide antigen, in any of the embodiments described above. According to the method, the activated antigen presenting cell is effective to activate a T-cell to produce a multivalent cellular immune response that is substantially higher than that produced by antigen presenting cells contacted with the selected polypeptide antigen alone. In
25 a preferred embodiment, the antigen presenting cell is a dendritic cell, isolated as described herein.

In yet another related aspect, the invention includes a method of inducing a cytotoxic T-cell response in a vertebrate subject. According to this aspect of the invention an isolated dendritic cell is contacted with an immunostimulatory polypeptide complex according to any of the
30 embodiments described above for a period of time effective to activate the antigen presenting cell. The antigen presenting cell is then injected into the mammalian subject. In a preferred embodiment, the antigen presenting cell that is activated and injected is a dendritic cell.

In further related aspects, the invention also includes the polypeptide complexes formed as described above. As described above, such polypeptide complexes are preferably formed

from a dendritic cell binding protein, preferably GM-CSF, and a polypeptide antigen. The polypeptide antigen is preferably a tissue-specific tumor antigen such as prostatic acid phosphatase (PAP), or an oncogene product, such as Her2, p21RAS, and p53; however, other embodiments, such as viral antigen antigens, are also within the contemplation of the invention.

5 In further related aspects, the invention also includes expression vectors and expression systems for producing the above-described immunostimulatory fusion proteins, as well as substantially purified nucleic acid molecules that encode such fusion proteins. In preferred embodiments, the nucleic acid molecules code for fusion proteins consisting essentially of GM-CSF and prostatic acid phosphatase or of GM-CSF and Her2.

10 The invention also includes a novel prostate carcinoma cell line, HLA A2.1 cells, that can be used a target cell for testing tumor cell killing, as described herein.

Brief Description of the Figures

FIG. 1 shows nucleic acid (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID
15 NO: 2) of PAP-GM-CSF fusion protein having as a peptide linker gly-ser resulting from the Bam HI linker (underlined);

FIG. 2 shows the amino acid sequence of the fusion protein Human Prostatic Acid Phosphatase/Human GM-CSF, with the PAP signal sequence not present in the mature protein shown in small caps, potential N-glycosylation sites marked "C", and potential disulfide bridges
20 marked "S-S";

FIGS. 3A and 3B show schematic representations of PAP-GM expression vectors pCEP4 PAPGM (3A) and PAPHGM-BAC (3B) used in mammalian (293 cells) and insect (SF21) cell lines, respectively;

FIG. 4 shows a graph of GM-CSF bioactivity of mammalian and baculovirus-derived PAP-
25 GM-CSF fusion proteins;

FIG. 5 shows a graph of acid phosphatase bioactivity of PAP-GM-CSF fusion proteins;

FIG. 6 shows a graph of lysis of prostate carcinoma cells by CTL primed with PAP-GM-CSF and stimulated with PAP-GM-CSF pulsed antigen presenting cells;

FIG. 7 shows a bar graph depicting blockade by HLA-class I blocking antibody of lysis
30 of prostate carcinoma cells by PAP-GM-CSF primed CTL;

FIG. 8 shows the nucleic acid (SEQ ID NO: 3) and amino acid (SEQ ID NO: 4) sequences of a GM-CSF-Her2 fusion protein in accordance with the present invention;

FIG. 9 shows titration of GM-CSF activity in crude Baculovirus Insect Cell (BVIC) culture supernatants from ratPAP or ratPAP-mouseGM-CSF infected Sf21 cultures as compared to

recombinant mouse GM-CSF and resultant proliferative response of rodent GM-CSF-dependent cell line GM-NFS-60;

FIG. 10 shows an immunization scheme of COP rats with purified dendritic cells pulsed with ratPAP-ratGM-CSF;

5 FIG. 11 shows nucleic acid and deduced protein sequence of the p53-GM-CSF fusion gene, where the Xba I linker that codes for serine and arginine is boxed; and

FIG. 12 shows the amino acid sequence of p53-GM-CSF fusion protein with the synthetic serine-arginine linker printed in boldface and underlined.

10 Detailed Description of the Invention

I. Definitions

As used herein, the term "tissue-specific antigen" refers to an antigen that is characteristic of a tissue type, including specific tumor tissues. An example of a tissue-specific antigen expressed by a tumor tissue is the antigen prostatic acid phosphatase, which is present in over
15 90% of all prostate tumors. By way of contrast, B-cell lymphomas produce immunoglobulin antigens that are particular to the individual tumor. Such particular tumor antigens are not considered to fall within the definition of the term "tissue-specific antigen."

The term "oncogene product" refers to any protein coded for by a gene associated with cellular transformation. Examples of oncogene products include, for example, Her2, p21RAS,
20 and p53.

"Antigen presenting cells" (APC) are cells that are capable of activating T cells, and include, but are not limited to, certain macrophages, B cells and dendritic cells.

"Potent antigen presenting (PAP) cells" are cells which, after being pulsed with an antigen, can activate naive CD8⁺ cytotoxic T-lymphocytes (CTL) in a primary immune response.

25 The term "dendritic cell" refers to any member of a diverse population of morphologically similar cell types found in lymphoid or non-lymphoid tissues. These cells are characterized by their distinctive morphology, high levels of surface MHC-class II expression (Steinman, et al., Ann. Rev. Immunol. 9: 271 (1991); incorporated herein by reference for its description of such cells). These cells can be isolated from a number of tissue sources, and conveniently,
30 from peripheral blood, as described herein.

The term "dendritic cell binding protein" refers to any protein for which receptors are expressed on a dendritic cell. Examples include GM-CSF, IL-1, TNF, IL-4, CD40L, CTLA4, CD28, and FLT-3 ligand.

II. Immunostimulatory Polypeptide Complexes

A. Selection of Components of the Polypeptide Complex

An immunogenic polypeptide formed in accordance with the present invention is generally characterized as an isolated polypeptide antigen which is covalently linked to a dendritic cell-binding protein.

1. Polypeptide Antigens

As stated above, isolated polypeptide antigens do not generally stimulate activation of T-cells *in vivo* or *in vitro*. It is the discovery of the present invention that certain types of polypeptide antigens, when coupled to a dendritic cell-binding proteins, such as those discussed below, stimulate T-cell activation.

The present invention identifies as particularly useful in this capacity (1) tissue-specific tumor antigens and (2) oncogene product peptide antigens. In the context of the present invention, the term "tissue-specific tumor antigens" refers to antigens that are common to specific tumor types. By way of contrast, antigens that are specific to a particular individual tumor, such as the B cell lymphoma tumor-associated idiotype antigen, are distinguishable from tissue-specific tumor antigens in that they have a characteristic epitope that varies from individual to individual. Such antigens are less useful in the context of the present invention, since a immunostimulatory reagents must be tailored to the individual tumor, and consequently do not form part of the invention.

Malignant tumors express a number of proteins that can serve as target antigens for an immune attack. These molecules include but are not limited to tissue-specific antigens such as MART-1, tyrosinase and GP 100 in melanoma and prostatic acid phosphatase (PAP) and prostate-specific antigen (PSA) in prostate cancer. Other target molecules belong to the group of transformation-related molecules such as the oncogene HER-2/Neu/ErbB-2. Yet another group of target antigens are onco-fetal antigens such as carcinoembryonic antigen (CEA). In B-cell lymphoma the tumor-specific idiotype immunoglobulin constitutes a truly tumor-specific immunoglobulin antigen that is unique to the individual tumor. B-cell differentiation antigens such as CD19, CD20 and CD37 are other candidates for target antigens in B-cell lymphoma. Some of these antigens (CEA, HER-2, CD 19, CD20, idiotype) have been used as targets for passive immunotherapy with monoclonal antibodies with limited success.

Thus, examples of tissue-specific tumor antigens include, but are not limited to prostatic acid phosphatase (PAP; associated with prostatic tumors), Melan-A/MART-1 (associated with melanoma; Coulie et al., 1994, J.Exp.Med. 180:35, Hawakami et al., 1994, PNAS 91:3515,

Bakker et al., 1994, J.Exp.Med. 179:1005), tyrosinase/albino (associated with melanoma; Kawakami et al., 1994, J.Exp.Med.), CD19, CD20 and CD37 (associated with lymphoma).

Likewise, oncogene product peptide antigens have been identified that are common to specific tumor types. These polypeptides can be incorporated into polypeptide complexes of the present invention as reagents that can be used generally to stimulate T-cell responses effective to react with tumors bearing such antigens. Oncogene product peptide antigens include but are not limited to HER-2/*neu* (Beckmann et al., 1992, Eur.J.Cancer 28:322) associated with human breast and gynecological cancers, and carcinoembryonic antigen (CEA) associated with cancer of the pancreas.

10 A variety of tumor markers are known in the art or are commercially available and include, but are not limited to the tissue-specific antigens that include cytokeratins, prostate-specific antigen (Wang et al., 1977), gp75/*brown* (Brichard et al., 1993, J.Exp.Med. 178:489) associated with melanoma, melanotransferrin (Real et al., 1984, J.Exp.Med. 160:1219), MUC1 (Barnd, 1989, PNAS USA 86:7159 and Vijayasaradhi et al., 1990, J.Exp.Med. 171:1375) associated with pancreas and breast cancer; oncogene/tumor suppressor genes that include EGF-R (Osborne et al., 1980), estrogen receptor, progesterone receptor, retinoblastoma gene product, myc associated with lung cancer, *ras*, p53, nonmutant associated with breast tumors, MAGE-1,3 (van der Bruggen et al., 1991, Science 254:1643 and Gaugler et al., 1994, J.Exp.Med. 179:921) associated with melanoma, lung, and other cancers.

20 Isolated viral antigens may include HIV antigens gp120, gp41, gag, RT, NEF, VIF; influenza antigens HA, core and matrix;; EBV antigens: EBNA, BFLF1, BOLFI, BGLF2, LMP2a, LMP2b, BBRF1, BBRF2, and P11L27; and human papilloma virus.

Polypeptide antigens such as those described above can be isolated, synthesized or recombinantly expressed according to methods known in the art. In most cases, DNA coding sequences have been identified for these molecules. In addition, many of the so-called "tumor markers" are available commercially. Such isolated antigens can be complexed with a dendritic cell binding protein chemically, as discussed below, or fusion protein constructs may be produced recombinantly, according to methods well known in the art.

As an example of the foregoing, prostatic acid phosphatase (PAP) is the prostate-specific isozyme of the ubiquitous enzyme acid phosphatase. PAP is a secreted molecule that has been identified as a serum tumor marker that is specific for prostate cancer. (Vihko, et al., FEBS Lett. (1988), 236, 275-281, Solin, et al., Biochem.Biophys.Acta (1990), 1048, 72-77). There is no evidence from the literature that PAP by itself might serve as an inducer and target of CTL. As is demonstrated below, the present invention shows that PAP can serve both as an

inducer of CTL and as a target in prostate cancer cells, when combined with the dendritic cell binding protein GM-CSF and used to stimulate antigen presenting cells (exemplified by dendritic cells) that are then used to prime CTL.

2. Dendritic cell binding proteins

5 The second component of the polypeptide complex of the present invention is a dendritic cell binding protein. As mentioned above, without relying on any particular mechanistic theory, it is believed that the presence of such a molecule in covalent complex with a protein antigen facilitates transport of the antigen over the plasma membrane of the antigen presenting cell, and more particularly, the dendritic cell, in a receptor-mediated, non-disruptive
10 way. It is further believed that the dendritic cell binding portion of the fusion protein serves to preserve the viability and functionality of the APC.

 An example of a dendritic cell binding protein is granulocyte-macrophage colony stimulating factor (GM-CSF). This glycoprotein, which has an apparent molecular weight of about 23-33,000 by SDS-PAGE, is a cytokine that has pleiotropic function both in hema-
15 topoiesis as well as in immunology. Both human and murine GM-CSF are synthesized with a 17-amino acid hydrophobic leader sequence that is proteolytically cleaved during secretion. The mature proteins are 127 (human) or 124 (murine) amino acids, and have core polypeptide molecular weights of 14,700 and 14,400, respectively, but share only 52% amino acid identity. The factor has been found to play a stimulatory role in the differentiation and survival of
20 dendritic cells and is active in both glycosylated and de-glycosylated forms.

 Human and murine GM-CSF have been shown to bind to both high affinity ($K_D=20-60$ pM) and low affinity ($K_D=1-6$ nM) binding sites on cells of the monocyte-macrophage, neutrophil and eosinophil cell lineages. Competition for binding by another member of the hemopoietic colony stimulating factors, Multi-CSF, has been shown when the binding is carried
25 out at 37°. Binding of GM-CSF to high affinity receptors results in rapid internalization and degradation of GM-CSF (Metcalf, D. and Nicola, N.A. (1995) The Hemopoietic Colony-Stimulating Factors, Cambridge University Press, NY.). These properties may be used to serve as a guide to the selection of additional dendritic cell binding proteins useful in forming immunostimulatory polypeptide complexes in accordance with the present invention.

30 B. Formation of Polypeptide Complexes

 Polypeptide complexes can be formed by chemical means, such as by conventional coupling techniques known in the art. For example, the peptides can be coupled using a dehydrating agent such as dicyclohexylcarbodiimide (DCCI) to form a peptide bond between the two peptides. Alternatively, linkages may be formed through sulfhydryl groups, epsilon

amino groups, carboxyl groups or other reactive groups present in the polypeptides, using commercially available reagents. (Pierce Co., Rockford, IL).

Polypeptide complexes can also be formed recombinantly as fusion proteins according to methods known in the art. Example 1 details the methods used to produce a GM-CSF-PAP fusion protein in accord with the present invention. Briefly, human PAP was cloned from a prostate carcinoma cell line according to methods known in the art. The stop codon at the 3' end of the sequence was mutated away, and a Bam HI site inserted in its place, to fuse the PAP cDNA to GM-CSF DNA. GM-CSF DNA was cloned from a PBMNC library according to standard methods. A Bam HI site was inserted at the 5' end of the DNA, and an XbaI cloning site was inserted at the 3' end, along with an in-frame stop codon. PCR-generated cDNA's were digested with appropriate restriction enzymes and cloned into restriction vectors for transfection into specific mammalian or insect cell lines. FIG. 1 shows nucleic acid and deduced amino acid sequences of the PAP-GM-CSF fusion polypeptide having a gly-ser peptide linker. FIG. 2 further illustrates the fusion protein sequence with potential glycosylation sites indicated as "C" and probable disulfide bridges shown as "S-S." FIGS. 3A and 3B show schematic representations of the PAP-GM-CSF expression vectors used for transfecting mammalian (293) and insect (SF21) cell lines, respectively.

The fusion expression vectors were used to transfect COS cells (transient expression) as well as mammalian 293-EBNA cells (Invitrogen) and insect SF21 cells (Clontech, Palo Alto, CA). Fusion protein products were recovered from the tissue culture supernatants, and affinity purified by passage over an anti-human PAP monoclonal antibody immunoaffinity column. Analysis by SDS-PAGE revealed protein bands migrating at 75 kD and 64 kD as products from mammalian and insect cells, respectively. The 75 Kd band corresponds to a size that is approximately 19.5 Kd larger than the predicted size of the PAP-GM-CSF polypeptide backbone which is 55.5 Kd. This can be explained by the presence of 5 potential N-glycosylation sites in the sequence, glycosylation at which would increase the apparent M_r of the protein, and is consistent with the fact that 293-EBNA cells contain fully functional human glycosylation machinery. The insect cell-derived fusion protein was approximately 8.5 Kd larger than the PAP-GM-CSF peptide backbone. These data are consistent with the known glycosylation patterns in Sf21 cells, which are reported to utilize N-glycosylation sites but which only add truncated carbohydrates that typically end with the addition of a single mannose residue.

The fusion molecules were tested for PAP and GM-CSF bioactivities in appropriate assays, detailed in Example 2. Both insect and mammalian cell-derived fusion proteins exhibited GM-

CSF activity, as evidenced by their ability to support growth of GM-CSF dependent cell lines (FIG. 4). Similarly, both products exhibited PAP activity (FIG. 5).

Fusion proteins constructed to incorporate oncogene product antigens are exemplified by incorporation of the oncogene product Her2. Her2 is a growth factor receptor belonging to the EGF-R family of receptors. It is over-expressed by breast cancer cells, ovarian cancer cells and a variety of other cancer cells. The cDNA coding for the extracellular domain of Her2 is cloned from a breast cancer cell line and fused to the GM-CSF cDNA, essentially as detailed for PAP-GM-CSF, above. Production of the soluble protein can be verified using Her2-specific monoclonal antibodies in an ELISA test, according to methods well-known in the art.

10 The fusion protein includes the sequences for the extracellular domain (amino acids 1-652) of Her2 (GenBank) and GM-CSF (FIG. 8). In this particular fusion protein the two proteins are linked by a leucine/glutamic acid linker which is generated by inserting a XHO I site.

The cellular tumor suppressor gene p53 is cloned and fused to GM-CSF, as described in Example 7. The sequence of the recombinant fusion gene is shown in FIG. 11, and the sequence of the produced polypeptide p53-GM-CSF is shown in FIG. 12. The p53-GM-CSF fusion protein is used to generate anti-p53 immunity as described for the induction of anti-PAP immunity by PAP-GM-CSF in Examples 3, 4 and 6.

15

Other oncogene product antigens are similarly incorporated into fusion proteins according to the methods described herein, using published sequences. In addition, other antigens, such as viral antigens, may be part of a fusion construct, according to the methods described herein.

20

While the foregoing description describes particular embodiments of the present invention, it will be appreciated that persons skilled in the art can substitute different antigens, vectors and expression cell lines, according to known methods, to prepare immunostimulatory polypeptide complex compositions in accordance with the principles described above. In addition, it is appreciated that the invention may also be practiced by inserting between the dendritic cell binding protein and the polypeptide antigen, a linker peptide or protein such as ubiquitin, according to recombinant methods known in the art.

25

III. Stimulation of T-cells

An important aspect of the present invention is the utility of the above-described polypeptide complex constructions in a method to target the antigen protein partner to Antigen Presenting Cells (APC), such as the cell type known as the "dendritic cell", described above. In accordance with the invention, the targeting occurs in a manner that results in entry into the class I pathway of antigen processing. The APC is then used to prime CTL *ex vivo* and *in vivo*, according to the methods discussed below.

30

Dendritic cells are highly potent APC's, and are the only APC that can prime naive CTL. While dendritic cell precursors present in human blood can take up antigen, they do not function as potent APC's. On the other hand, the mature dendritic cell is the most potent APC, but it does not take up antigen spontaneously *in vitro*. In the past, it was necessary to treat
5 mature dendritic cells with physical force (liposomes, osmotic shock) or to coat them with exogenous small (8-11 amino acids in length; generally 9-mer) peptide antigens to enable them to act as APC.

The present invention enables introduction of an exogenously added protein into the class I pathway of a mature dendritic cell. Such induction can be effected *in vitro*, by isolating
10 APC's such as dendritic cells, "pulsing" or contacting them with the polypeptide complex for an extended period of time, then using the pulsed APC's to stimulate autologous T-cells *in vitro* or *in vivo*. In the latter case, the pulsed APC's are administered (approximately 10^7 cells/injection) to the subject. The response of the subject is measured by monitoring the induction of a cytolytic T-cell response, a helper T-cell response and antibody response towards the
15 antigen in peripheral blood mononuclear cells by methods well known in the art. Multiple doses may be administered to produce an adequate response.

The use of GM-CSF fusion antigen-stimulated dendritic cells yields superior results to other approaches, such as the peptide pulsed dendritic cell preparations. That is, while dendritic cells pulsed with 8-11-mer peptides are able to induce immunity, such immunity is
20 directed to a single T-cell epitope. Proteins incorporated into liposomes or delivered to the dendritic cells by osmotic shock induce reactivity towards multiple T-cell epitopes; however, this process is relatively ineffective due to the inherent toxicity of these treatments to dendritic cells.

In contrast, GM-CSF fusion antigens of the present invention induce immunity towards
25 multiple epitopes and preserve and enhance at the same time viability and function of the dendritic cell. In practice, the compositions of the present invention are found to induce a cellular (T-cell) activation that is multivalent and substantially higher than that produced by a selected antigen alone.

In experiments carried out in support of the present invention, the fusion protein consisting
30 of PAP and GM-CSF described in the previous section was used for *in vitro* introduction to dendritic cells and subsequent activation of cytolytic T cells, as detailed in Example 4. Briefly, HLA-A2.1 positive PBMNC were isolated by standard methods and primed with the fusion protein for 2-5 days. The cell mixture was depleted of CD4⁺ T-cells, separated into high and low density fractions, and the separate cultures were re-stimulated weekly with autologous PAP

GM-CSF pulsed APC's. Lytic potential of the T-cells present in the fractions was assessed using a standard chromium release assay using an HLA-A2.1-transgenic prostate carcinoma cell line as target. This novel cell line was constructed according to the methods detailed in Example 3 herein and is useful in screening and analysis of HLA class I restricted cytotoxic T-lymphocytes.

Results of the lysis assays are presented in FIG. 6. As shown, of the four different T-cell cultures tested, three displayed substantial dose-dependent cytotoxicity towards the prostate carcinoma target. The highest degree of cytotoxicity was effected by cells that fractionated into the high density pellet fraction on day 5 (open circles, 5-day P). High (open squares, 2-day P) and low (closed squares, 2-day IF) density cells primed for two days showed roughly equal potency. Cell cultures derived from day 5 low density interface fraction (closed circles, 5-day IF) displayed little or no cytotoxicity.

FIG. 7 shows that the tumor-specific cytotoxicity was substantially reduced in the presence of the HLA class I-specific blocking antibody W6/32 at an Effector:Target (E/T) ratio of 10:1 and is completely eliminated by the antibody at an E/T ratio of 3.3/1. Control antibody CA 141 did not reduce T-cell mediated killing. These experiments demonstrate that the interaction with the target cell is mediated by via classical T-cell receptor/HLA-class I restricted antigen-specific pathway.

The foregoing results demonstrate the efficacy of fusion polypeptide complexes formed in accordance with the present invention in stimulating T-cell responses *in vitro*. These responses can be compared to those stimulated by the antigen alone (in the absence of the dendritic cell binding protein). In addition, their multivalent character can be tested by standard methods.

IV. Therapeutic applications

A. *In vitro/ex vivo* Therapy

The present invention provides for induction by isolated or soluble proteins of cellular immunity *in vitro* by presenting a specific antigen to an antigen presenting cell (APC), such as a dendritic cell, as part of an immunogenic fusion protein. As discussed above such induction is not generally observed *in vitro* using soluble, whole antigens as induction materials.

In practice, dendritic cells are isolated from an individual, using known methods, or preferably, as described in Example 5. The dendritic cells (or other APC's) are mixed with 10 ng/ml equivalent of GM-CSF fusion antigen, as described in Example 4. The cell preparation may then be depleted of CD4⁺ T-cells by solid phase immunoadsorption and further fractionated to enrich for cells having cytolytic activity. Doses of about 10⁷ cells are then administered to the subject by intravenous or central injection according to established

procedures (e.g., infusion over 30 to 60 minutes). The responsiveness of the subject to this treatment is measured by monitoring the induction of a cytolytic T-cell response, a helper T-cell response and antibody response towards the antigen in peripheral blood mononuclear cells by methods well known in the art.

- 5 In addition to the direct *in vivo* administration regimen described above, the APC cells can be used, for example, in *ex vivo* somatic therapy, *in vivo* implantable devices and *ex vivo* extra-corporeal devices. They can also be employed in the screening of antigenicity and immunogenicity of peptide epitopes from tumor- and virus-specific antigens.

- 10 In certain cases, it may be advantageous to use cells obtained from one individual to treat a condition in a second individual. For example, HIV-infected individuals with AIDS are often not able to mount anti-viral T-cell responses. In such cases, CTL can be isolated from healthy HLA-matched individuals, such as siblings, be stimulated or primed with antigen-pulsed DC *in vitro*, expanded, and administered back to the HIV-infected individuals.

B. *In vivo* Therapy

- 15 The fusion protein compositions described herein can also be administered directly to an individual as a vaccine, in order to stimulate the individual's cellular immunity pathways *in vivo*. Here a dose of about 5 to 200 microgram/kg fusion protein, is administered, preferably at days 0, 14 and 28 with an optional boosting dose at 6 months. The response of the subject is measured by monitoring the induction of a cytolytic T-cell response, a helper
20 T-cell response and antibody response towards the antigen in peripheral blood mononuclear cells by methods well known in the art.

C. Results from Experimental Models of Immunostimulation

- Example 6A provides details of the construction of fusion antigens and injection protocols used in an experimental rat model of immunostimulation. A fusion protein made up
25 of rat PAP and rat GM-CSF was injected directly into rats (*in vivo* stimulation) or to stimulate rat dendritic cells for later injection into rats (*ex vivo* induction). It is necessary to use rat molecules and cells, since human effector cells do not function normally in other species and are, in addition, antigenic in other species, such as rat. Moreover, the target antigen (PAP) is different between humans and other species, and human GM-CSF would not be expected to
30 produce the same type of effect in other species. Nonetheless, it is understood that the use of rat cells in rats should provide a model system for the use of human cells and proteins in humans.

As detailed in Example 6, rat prostatic acid phosphatase (ratPAP) was prepared recombinantly and was fused to rat GM-CSF. Dendritic cells from rat splenic tissue were

pulsed with ratPAP-ratGM-CSF and were injected into normal rats. In addition, alternate delivery systems were tested, since autologous PAP-directed immunization has not previously been carried out, to the best of our knowledge.

In studies carried out in support of the present invention and detailed in Example 6B, rats were immunized with either (i) ratPAP-ratGM-CSF pulsed dendritic cells, (ii) ratPAP-ratGM-CSF alone, or (iii) rat PAP in conventional CFA adjuvant. Rat organs (prostate plus six major organ systems) were later examined for immunopathology evoked by autologous PAP-directed immunization. According to the rat model system, it was expected that a successful immune response would result in immune targeting of PAP-expressing organs, and in particular, the prostate.

As shown in Table 1, the histopathology studies show that immunizations with rat PAP-rat GM-CSF pulsed dendritic cells or with rat PAP rat GM-CSF fusion protein induce organ-specific autoimmunity that is limited to the prostate. In contrast, immunization with ratPAP/CFA protein did not produce this result. This result demonstrates that PAP-GM-CSF itself and PAP-GM-CSF pulsed dendritic cells produce antigen-specific autoimmunity that is significantly better than, and in fact, cannot be produced by, the antigen alone in association with a conventional adjuvants.

Table 1

Organ Histopathology^a of immune and non-immune rats

Rat No. ^b	Brain	Lung	Heart	Liver	Kidney	Colon	Prostate	Comments, Prostate
1C	N	N	N	N	N	N	N	N
2C	N	N	N	N	N	N	N	N
3C	N	N	N	N	N	N	N	N
4C	N	N	N	N	N	N	N	N
5T	N	N	N	N	N	N	3	Moderate subacute multifocal interstitial prostatitis
6T	N	N	N	N	N	N	2	Mild scattered inflammation in the interstitium
7T	N	N	N	N	N	N	1	Trace to scattered inflammation in the interstitium
8T	N	N	N	N	N	N	2	Mild multifocal inflammation in the interstitium
1 CFA	ND	ND	ND	ND	ND	ND	N	
2 CFA	ND	ND	ND	ND	ND	ND	N	

1 PAP GM	ND	ND	ND	ND	ND	ND	1	Mild multifocal lymphocytic inflammation in the interstitium
2 PAP GM	ND	ND	ND	ND	ND	ND	1	Mild multifocal lymphocytic inflammation in the interstitium

* Pathology grading: ND = Not evaluated; N = No significant lesions; 1 = trace/mild; 2 = mild; 3 = moderate; 4 = severe.

* Immunization groups: animals 1C, 2C, 3C, 4C were non-immunized controls; animals 5T, 6T, 7T, 8T were immunized with rat PAP rat GM-CSF pulsed dendritic cells; animals 1CFA, 2CFA were immunized with rat PAP immersed in CFA; animals 1 PAP GM, 2 PAP GM were immunized with recombinant rat PAP rat GM-CSF.

The following examples illustrate, but in no way are intended to limit the invention.

Example 1

Construction of PAP/GM-CSF Fusion Proteins

If not described otherwise, general cloning procedures were performed according to standard techniques as described in Sambrook, et al., Molecular Cloning: A Laboratory Manual (1989).

Human PAP was cloned from the prostate carcinoma cell line LnCaP.FGC (American Type Culture Collection, Rockland Maryland; "ATCC"). Synthetic oligonucleotide primers containing the sequence CGGCTCTCCTCAACATGAGAGC were custom synthesized according to standard methods by Keystone Labs (Menlo Park, CA). These primers are homologous to the 5' end of the known PAP cDNA sequence which is published in the GenBank database.

Hind III, Mun I or Xho I restriction sites were attached, according to the requirements of the particular expression vector used. On the 3' end an oligonucleotide of the sequence CACAGGATCCATCTGTACTGTCTCAGTACC was constructed that mutated away the stop codon 387 of the PAP sequence and substituted for it a Bam HI site that codes for amino acids glycine and serine. This Bam HI site was used to fuse the PAP cDNA to the GM-CSF cDNA, which was cloned from peripheral blood mononuclear cells (PBMNC), based on the sequence which is published in GenBank. At the GM-CSF 5' end a Bam HI site was attached to an oligonucleotide GACTGGATCCGCACCCGCCGCTCGCCC that is homologous to the nucleotides that code for amino acids 18-23 in the GM-CSF sequence. The 3' end of GM-CSF was generated with a an oligonucleotide GATCTCTAGAGCTTGGCCAGCCTCATCTGG that ends after the in frame stop of GM-CSF and creates an Xba I cloning site. Poly A+ RNA was isolated from cell line LnCaP.FGC and from PBMNC with the Micro Fast track kit (Invitrogen) according to the manual supplied by the manufacturer. The Poly A+ RNA was

then reverse transcribed with the cDNA cycle kit (Invitrogen) according to procedures described in the accompanying manual. First strand cDNA was then subjected to 25 cycles of polymerase chain reaction (PCR) with the above described primers. The conditions on a Perkin-Elmer thermal cycler 9600 were as follows. 25 cycles of amplification were performed at: Denaturation: 94°C, 15 seconds; annealing 55°C, 15 seconds; extension 72°C, 60 seconds. The 25 cycles were followed by a final extension period of 420 seconds at 72°C. The PCR products were analyzed on agarose gels. They were digested with the appropriate restriction enzymes and cloned into the vectors pCR3 (Invitrogen) to create pCR3-PAP-GM (not shown), pCEP 4 (Invitrogen) to create pCEP4-PAP GM (FIG. 3A) and into pBacPac 8 (Clontech) to create PAPHGMBAC (FIG. 3B). The DNA sequences of the cloned constructs were confirmed using standard methods on a fluorescent sequencer Model ABI 373A (Applied Biosystems, Foster City, CA). The nucleotide sequence and the deduced amino acid sequences are presented as SEQ ID NO.: 1 and SEQ ID NO.: 2, in FIG. 1, respectively.

pCR3 PAP-GM was electroporated into COS-7 cells (ATCC) for transient expression experiments. After it was confirmed that a protein of the predicted size, immunological identity and function could be expressed transiently in COS-7 and 293-EBNA (Invitrogen) cells stable transfectants were generated in the human embryonic kidney cell line 293-EBNA, using an episomal expression vector pCEP4 (Invitrogen, San Diego, CA). After electroporation and selection in hygromycin, recombinant clones were generated by plating the cells under limiting dilution conditions and screening for PAP bioactivity in the cellular tissue culture supernatants. The highest producing clones were adapted to protein-free media and grown in CellMax hollow fiber bioreactors (Gibco, Gaithersburg, MD). Spent media from the cultures were collected, pooled and clarified by centrifugation. They were then passaged over an immunoaffinity column that was made by coupling the human PAP-specific monoclonal antibody ATCC HB8526 (ATCC) to a Sepharose resin. After washing, the bound material was eluted at low pH, neutralized and dialyzed against physiological buffer. The eluted fraction was analyzed by denaturing SDS-PAGE electrophoresis under reducing conditions. The resulting gel showed a single protein band at 75 kD which corresponds to the predicted size of fully glycosylated PAP-GM-CSF.

PAPHGM-BAC was also used to generate a recombinant *Autographa californica* nuclear polyhedrosis virus (AcNPV, baculovirus) by homologous recombination of PAPHGM-BAC with BacPAK6 viral DNA (Clontech, Palo Alto, CA). Reagents were used from the BacPAK baculovirus expression system (Clontech) and procedures were carried out essentially as described in the product manual. PAPHGM-BAC and BacPAK6 were co-transfected into SF21

cells (Clontech) by lipofection. The spent tissue culture supernatant was collected on day 5. It was titered onto fresh SF21 cells which were then grown in semisolid media for another 4 days. After the monolayers were stained with neutral red, viral plaques were identified and picked with a Pasteur pipet. Recombinant plaque-purified virus was eluted into fresh media and was then used to screen for production of PAP-GM-CSF in fresh SF21 cells. Positive plaques were identified and used to generate viral stocks and recombinant protein in subsequent rounds of infecting fresh SF21 cells. The media of production cultures were collected three days after infection. They were then processed as described for PAP-GM-CSF that was derived from 293-EBNA cells. Analysis of the immunoaffinity-purified protein revealed a single protein band at 64 kD after silver staining of an SDS-PAGE gel.

Example 2 Bioactivity of PAP-GM-CSF fusion proteins

PAP-GM-CSF fusion proteins from all expression systems described in Example 1 were analyzed for their ability to support the growth of GM-CSF dependent cell lines. They were also analyzed for enzymatic activity in acid phosphatase assays. Standard bioassays were used to determine the GM-CSF bioactivity.

GM-CSF Activity. The GM-CSF dependent human erythroleukemia cell line TF-1 (ATCC, Rockville, MD) and the acute monocytic leukemia cell line AML-193 (ATCC) were used to analyze whether GM-CSF retains its bioactivity after fusion to PAP. The cell lines which are routinely cultured in GM-CSF-containing media were starved in regular media for 24 hours before the assay. They were plated at 1500 cells per well in triplicates in tissue culture medium. Test supernatants or recombinant GM-CSF as a positive control were added to the cells. Cells were cultured for 72 hours and were then pulsed for 4 hours with 1 microcurie tritiated thymidine per well to determine rate of DNA synthesis. FIG. 4 shows that both mammalian derived and insect cell-derived PAP-GM-CSF support the growth of GM-CSF dependent cell lines. The calculated relative bioactivity of PAP-GM-CSF is 20% of the activity of control recombinant GM-CSF dependent cell lines on a molar basis.

Acid Phosphatase Activity. The bioactivity of the second component of the fusion protein was determined in an enzymatic assay for acid phosphatase activity. Acid phosphatase was measured as the ability of the protein to hydrolyze para-nitrophenyl phosphate (pNPP) at acid pH. Briefly, the test liquid was diluted in 50mM sodium citrate pH 4.8. pNPP is added to a final concentration of 2 mg/ml. After 30 minutes incubation at 37°C, an equal volume of 1M NaOH was added to the reaction. Hydrolyzed pNPP under these conditions has a yellow color which can be quantified with a spectrophotometer at 405 nm. FIG. 5 shows that both

mammalian derived and insect cell derived PAP-GM-CSF hydrolyzed pNPP under acid conditions.

Thus, it is clear that the original biological activity of both PAP and of GM-CSF is conserved in PAP-GM-CSF fusion proteins.

5

Example 3
Generation of a target cell line
for HLA class I-restricted cytotoxic T-lymphocytes

If not otherwise described, all tissue culture techniques, cell manipulations and assays were performed according to standard techniques.

In order to generate a prostate cancer cell line that could be used as a target cell on a defined genetic background an HLA A2.1-transgenic prostate cancer cell line was generated. HLA A2.1 is the best studied restriction element in human HLA class I restricted immune responses and is the most frequent allele in Caucasians. A cDNA that codes for the published sequence (GenBank) of HLA A2.1 was isolated and cloned from the lymphoblastoid cell line JY. HLA A2.1 heavy chain cDNA was amplified with the sense primer 5-AgACgCCgAggAT-ggCC-3' and the anti-sense primer 5-CCTCTCTggAACAggAAAgATg-3'. Methods and conditions were as described for PAP and GM-CSF except that the cell line JY (obtained from Dr. Ed Engleman, Stanford University Blood Bank, Stanford, CA) was used as starting material. The resulting gene fragment was cloned into the pCR3 vector with the TA-cloning kit (Invitrogen). The prostate carcinoma cell line LnCaP.FGC (ATCC) was transfected with this expression plasmid which confers the expression of HLA A2.1. The parent cell line does not express the A2.1 allele. After drug selection in G418 (Gibco) the resulting transfectants were selected for HLA A2.1 expression by solid phase immunoadsorption ("panning") with a HLA A2.1-specific monoclonal antibody (BB7.1, ATCC). The resulting cell line homogeneously expressed HLA-A2.1 whilst its parent remained negative. This novel transgenic cell line is uniquely useful in the screening and analysis of HLA class I restricted cytotoxic T-lymphocytes.

30

Example 4
Induction of prostate cancer-specific CTL
by PAP-GM-CSF

A T-cell *in vitro* priming and expansion system was used to establish the utility of PAP-GM-CSF in the generation of HLA class I restricted CTL.

35

HLA-A2.1-positive PBMNC were isolated by standard methods on density gradient (FICOLL-HYPAQUE, Pharmacia Fine Chemicals, Piscataway, NJ) having a density of 1.077 gr/ml. The cells were primed with 10 ng/ml equivalent of PAP-GM-CSF for two or five days.

(GM-CSF potency equivalent was measured on a GM-CSF dependent cell line as detailed in Example 3; the actual weight used was twenty-fold higher because of the different size and specific activity of PAP-GM-CSF.) The cell preparation was then depleted of CD4+ T-cells by solid phase immunoadsorption and separated into low density and high density cells over a 1.068 gr/ml density gradient. The different fractions were then cultured separately in AIM V media (Gibco, Gaithersburg, MD) supplemented with rIL-2 (20 U/ml). Autologous PBMNC that were cultured in 20 ng/ml PAP-GM-CSF in Aim V media were used as antigen presenting cells for re-stimulation at weekly intervals. Lytic potential of the cells was assessed in a standard 4-hour chromium release assay with the HLA-A2-1-transgenic prostate carcinoma cell line LnCaP.FGC as a target.

FIG. 6 shows induction of Prostate-carcinoma LnCaP.FGC/A2.1-specific cytotoxic T-lymphocytes by PAP-GM-CSF pulsed antigen presenting cells. HLA-A2.1 positive PBL were primed with 10 ng/ml GM-CSF equivalents of PAP-GM-CSF for two or five days. The cultures were depleted of CD4+ T-cells and separated into low (IF) and high (P) density fractions over a Nycodenz density gradient having a density of 1.068 gr/ml.

To investigate whether the observed cytotoxicity was a HLA-class I-restricted CD8+ cytolytic T-cell mediated phenomenon a blocking assay with the monomorphic HLA class I-specific monoclonal antibody W6/32 (ATCC) antibody was performed. W6/32 blocks HLA class I mediated killing in standard assays, whilst control antibody CA141 is specific for HLA class II (DR) and will not interfere with class I restricted killing. T-cell cultures which were derived from the 5 day pellet fraction (described above) which displayed the highest cytotoxicity were used for this experiment. The T-cell lines used in experiment contained 38% CD3/CD8 positive T-cells. Their lytic potential was assessed in a standard 4-hour chromium release assay with the HLA-A2-1-transgenic Prostate carcinoma cell line LnCaP.FGC as a target. FIG. 7 shows that tumor-specific cytolysis was substantially reduced in the presence of the HLA class I blocking antibody W6/32 at an Effector:Target (E/T) ratio of 10:1 and is completely eliminated by the antibody at an E/T ration of 3.3/1. Control antibody CA 141 did not reduce T-cell mediated killing. These experiments demonstrate that the interaction with the target cell is mediated by via classical T-cell receptor/HLA-class I restricted antigen-specific pathway.

Example 5 Preparation of Dendritic cells

Buffy coats prepared from one unit of blood from HLA-A0201 positive volunteer healthy donors are obtained from the Stanford University Blood Center (Stanford, CA). Cells are

harvested from the leukopacs, diluted to 60 mL using $\text{Ca}^{++}/\text{Mg}^{++}$ free phosphate buffered saline (D-PBS; Gibco Laboratories, Grand Island, NY) and layered over two 15 mL columns of organosilanized colloidal silica (OCS) separation medium (prepared as described by Dorn in U.S. Patent 4,927,749, incorporated herein by reference, at a density 1.0720 gr/mL, pH 7.4, 280 mOsm/kg H_2O) in 50 mL centrifuge tubes, preferably cell-trap tubes. The OCS medium is preferably prepared by reacting and thus blocking the silanol groups of colloidal silica (approx. 10-20 nm diameter particles) with an alkyl trimethoxy silane reagent.

Related colloidal silicas and methods for production thereof are disclosed in U.S. Patent 4,927,749 to Dorn. In a preferred embodiment, the OCS density gradient material is diluted to an appropriate specific density in a physiological salt solution supplemented with polyvinylpyrrolidone (PVP) such as PVP-10 available from Sigma Chemical Co. (St. Louis, MO).

The tubes are centrifuged at $1000 \times g$ for 35 minutes at room temperature. The centrifuge run is allowed to stop without braking and the peripheral blood mononuclear cells (PBMC), present at the interface, are harvested.

PBMC are re-suspended in D-PBS, centrifuged once at $650 \times g$ for 10 minutes and twice more at $200 \times g$ for 5 minutes to remove platelets. Platelet-depleted PBMC are re-suspended in 60 mL of D-PBS, layered on top of two columns of 15 mL of OCS (density 1.0610 gr/mL, 280 mOsm/kg H_2O) in a centrifuge tube and centrifuged at $650 \times g$ for 25 minutes at 4°C without braking. The resulting interface (primarily monocytes) and pellet cells (primarily lymphocytes) are harvested and washed with D-PBS by centrifugation at room temperature (once at $650 \times g$ for 10 minutes and twice thereafter at $200 \times g$ for 5 minutes).

In instances where the dendritic cells are used to generate peptide-specific cytotoxic T lymphocytes (CTL) for purposes of elucidating their antigen presentation function, the interface fraction (mostly monocytes) is re-suspended in cold pooled human AB serum (Irvine Scientific, Santa Ana, CA) to which an equal volume of 80% AB serum 20% dimethyl sulfoxide (DMSO) (Sigma Chemical Company, St. Louis, MO) is added dropwise. The resulting cell suspension is aliquoted into cryovials and frozen in liquid nitrogen. The monocytes can be used for re-stimulation of CTL for expansion.

The pellet fraction is re-suspended in 100 mL of AB Culture Medium, inoculated into two T-75 tissue culture flasks and cultured in a humidified 5% CO_2 incubator for 40 hours. Following the incubation, the non-adherent cells are harvested by moderate pipeting, washed and re-suspended at a concentration of $2 - 5 \times 10^6$ cells/mL in AB Culture Medium. The cell suspension is layered onto four columns of 4.0 mL OCS separation medium (density 1.0565

gr/ml, pH 7.4, 280 mOsm/kg H₂O), in AB Culture Medium and centrifuged at 650 × g for 20 minutes at room temperature without braking.

The interface and pellet cells are harvested and washed in AB Culture Medium (Basal RPMI-1640 medium, Gibco Laboratories, Grand Island, NY) by centrifugation once at 650 × g for 10 minutes and twice thereafter at 200 × g for 5 minutes each at room temperature. The yield and viability of both cell fractions is estimated by counting on a hemocytometer using trypan blue exclusion.

The purity of dendritic cells in the interface fraction is quantified following analysis on a flow cytometer (FACS). Dendritic cells are characterized as negative for cell phenotype markers CD3 (T lymphocytes), CD14 (monocytes), CD16 (NK cells) and CD20 (B-cells) and positive for HLA class II expression using dual staining with HLA-DR (on the FITC channel) and a cocktail of CD3, CD14, CD16, CD20 (on the PE channel). Dual staining with IgG2a on both the FITC and PE channels can be used as isotype control.

The morphology of the cells can also be evaluated using photomicroscopy. The DC enriched fraction contains large sized veiled cells with cytoplasmic processes extending from the cell surface, features characteristic of DC.

Example 6

In vivo efficacy of PAP-GM-CSF-pulsed dendritic cells

A. Construction, expression and bioactivity of recombinant ratPAP and GM-CSF fusion proteins

Recombinant rat PAP-rat-GM-CSF was constructed as follows: rPAP cDNA was amplified from first strand cDNA made from mRNA isolated from rat prostrate (Harlan) using primers which delineate the fragment containing nucleotides 15-1177 (Genbank Acc. M32397) and which add an exogenous Xho I restriction site at the 5'-end and exogenous BamHI and Bln I sites at the 3'-end to facilitate insertion into the pBacPAK8 vector. In the absence of GM fusion, the Bln I site encodes an in-frame stop codon. Mature rat GM-CSF cDNA was amplified by PCR from first strand cDNA made from mRNA isolated from ConA-stimulated rat splenocytes using primers which delineate nucleotides 1-384 (GenBank Acc. U00620) and add an exogenous BamHI site at the 5'-end and an exogenous Xba I site at the 3'-end.

The ratPAP and ratPAP-ratGM-CSF plasmids were each mixed with linearized BV viral genome plasmid and the mixtures were transfected into SF21 cells using Lipofectin as supplied in a recombinant BV transfection kit (Clontech). Six days after transfection, the culture supernatants were collected and titrated on Sf21 monolayers under agarose to form viral plaques. Four days later the cells were stained with neutral red and candidate viral plaques

were picked and expanded on Sf21 cells to screen for recombinant BV using PAP enzymatic activity as a readout. PAP⁺ BV clones were chosen and expanded in SF21 large-scale suspension cultures for viral stocks and subsequently for protein production using protein-free Sf900 II media (Gibco/BRL).

5 All proteins exhibited PAP enzymatic activity as shown by hydrolysis of PNPP, the enzyme substrate used in a standard acid phosphatase assay and, in the case of GM-CSF fusion proteins, GM-CSF bioactivity as shown by bioassay on GM-NFS-60 cells. GM-CSF bioactivity of a representative fusion protein example, ratPAP-ratGM-CSF (FIG. 9), was determined in a GM-CSF rat cell proliferation assay carried out using the GM-CSF-dependent cell line
10 GM-NFS-60. Five thousand cells were plated per well in 200 μ l of media with the designated amounts of cytokine. Proliferation was determined by pulsing with 1 μ Ci [³H]thymidine per well during the final 4 hr of a 48 hr assay. Crude baculovirus culture supernatant or with 10ng/ml commercially available recombinant mouse GM-CSF (rmGM) was diluted and added to the cultured cells as indicated, and proliferation was measured.

15 RatPAP was expressed as a fusion protein with a tail of 6 histidine-residues (ratPAP(His₆)) attached to its C-terminus. It was purified by means of metal-chelate affinity chromatography (Ni-NTA columns), according to standard methods. A typical purification resulted in >90% pure recombinant protein. Purification of ratPAP-ratGM-CSF was performed by a combination of ion-exchange/hydrophobic interaction chromatography. This procedure typically yields
20 >50% pure rat PAP-GM-CSF.

B. Immunization of Animals

Male inbred rats were used in all studies that were designed to address the immunogenicity of ratPAP *in vivo*. The goal of such studies was to determine whether immunity towards the autoantigen rat PAP could be elicited.

25 Normal 8-week old male rats (Wistar or COP) were immunized with PAP in CFA (approx. 200 μ g) or with PAP-GM-CSF fusion protein (200 μ g). After conclusion of the immunization, the rats were euthanized and their prostates were examined histologically. An additional group of rats was treated with ratPAP-ratGM-CSF pulsed dendritic cells. Dendritic cells from rat spleens were isolated as outlined in FIG. 10, pulsed overnight with ratPAP-ratGM-CSF that
30 had been purified from baculovirus culture supernatant by a combination of cation-exchange chromatography, hydrophobic interaction chromatography and anion-exchange chromatography. Cells (5-10 x 10⁶ cells/rat) were injected back into syngeneic hosts. Two weeks after the third immunization, animals were sacrificed and prostate, brain, lungs, heart, liver, kidney and colon

were analyzed by a veterinarian histopathologist and compared to tissues from control animals which were not immunized.

Phenotype of rat dendritic cells was determined using flow cytometry. To determine their antigen-presenting capacity, cells were used as stimulators in an allogeneic mixed lymphocyte reaction and their allostimulatory capacity to total splenocytes and to adherent splenocytes was compared. The typical range for dendritic cell purities obtained by this method is generally between 30% and 80%. The antigen-presenting capacity of dendritic cells from the rats was analyzed by using them as stimulators in an allogeneic mixed lymphocyte reaction with SD rat splenocytes as responders. Their allostimulatory capacity was compared to total splenocytes and to adherent splenocytes from both COP and SD strains.

SD-splenocytes were not stimulated by syngeneic SD-splenocytes, adherent splenocytes or dendritic cells. When allogeneic COP stimulators are used, the dendritic cell fraction stimulates several orders of magnitude stronger than spleen cell or adherent spleen cells from COP rats.

Histopathology of immunized rats. Histopathology was performed by a consulting veterinary histopathologist according to standard procedures. Animals were surveyed for histopathological changes to prostate, brain, lungs, heart, liver, kidney and colon, as summarized in Table 1. All organs in all control animals (1C, 2C, 3C, 4C) showed no significant change. All organs except the prostates in the treated animals showed no significant change. All animals treated with rat PAP rat GM-CSF pulsed dendritic cells suffered from interstitial prostatitis. The intensity varied: one animal (7T) had grade 1 (= trace) lesions, two animals (6T, 8T) had grade two lesions (= mild) and one animal (5T) had grade 3 (= moderate) lesions. Animals which were immunized with rat PAP in CFA (1CFA, 2CFA) did not show any histopathology of the prostate. Animals that were immunized with rat PAP rat GM-CSF protein (1 PAP GM, 2 PAP GM) had a grade 1 interstitial lymphocytic prostatitis.

Example 7 Construction and expression of p53-GM CSF fusion proteins

The cellular tumor suppressor gene p53 was cloned according to publicly known sequences (Genbank database release 91, entry HSP53) by means of polymerase chain reaction with the sense primer CgCggATCCTCACTgCCATggAggAgC and the antisense primer CTAgtCTAgA-CTCTgAgTCaggCCCTTCTgTC which includes an XBA I site that codes for a serine-arginine linker. GM-CSF was cloned as described in Example 1 except that primers CTAgtCTAgATC-TgCACCCgCCCgCTCgCCC (sense) and CCggAATTCTCAGTgATggTgATggTgATgCgATC-CTCTCATCTCCTggACTggCTCCCAgC (anti-sense) were used. The antisense primer codes

for the sequences MetArgGlySerHisHisHisHisHisHis which is attached to the C-terminus of GM-CSF and can be used for detection and metal-chelate affinity chromatography purification of the fusion protein. The resulting sequences are shown in FIGS. 11 and 12. Recombinant baculovirus transfer vector and recombinant baculovirus was generated as described in Example

- 5 1. When expressed in SF21 insect cells as detailed in Example 1 this baculovirus produces p53-GM-CSF fusion protein which is used to generate anti-p53 immunity as described for the induction of anti-PAP immunity by PAP-GM-CSF in Examples 3, 4 and 6. The sequence of the recombinant fusion gene is shown in FIG. 11. The sequence of the produced polypeptide p53-GM-CSF is shown in FIG. 12.

- 10 While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention.

IT IS CLAIMED:

1. A therapeutic composition, comprising an isolated potent antigen presenting cell effective to activate T-cells to produce a multivalent cellular immune response against a selected antigen,
5 at a T-cell activation level substantially higher than that produced by such a potent antigen presenting cells stimulated by the selected antigen alone.
2. The therapeutic composition of claim 1, wherein the potent antigen presenting cell is stimulated by exposing the cell *in vitro* to a polypeptide complex consisting essentially of a dendritic
10 cell-binding protein and a polypeptide antigen selected from the group consisting of a tissue-specific tumor antigen and an oncogene gene product.
3. The therapeutic composition of claim 2, wherein the dendritic cell-binding protein is GM-CSF.
15
4. The therapeutic composition of claim 2, wherein the polypeptide antigen is the tumor-specific antigen prostatic acid phosphatase.
5. The therapeutic composition of claim 2, wherein the polypeptide antigen is an oncogene
20 product peptide antigen selected from the group consisting of Her2, p21RAS, and p53.
6. The therapeutic composition of claim 2, wherein the polypeptide complex further comprises, between said dendritic cell-binding protein and said polypeptide antigen, a linker peptide.
25
7. The therapeutic composition of claim 2, wherein the potent antigen presenting cell is an activated dendritic cell.
8. A method of activating an isolated antigen presenting cell *in vitro*, comprising
30 contacting said isolated antigen presenting cell with a polypeptide complex consisting essentially of a dendritic cell-binding protein covalently linked to a polypeptide antigen selected from the group consisting of a tissue-specific tumor antigen, and an oncogene product peptide antigen,

wherein said activated antigen presenting cell is effective to activate a T-cell to produce a multivalent cellular immune response that is substantially higher than that produced by antigen presenting cells contacted with the selected polypeptide antigen alone.

5 9. The method of claim 8, wherein said dendritic cell-binding protein is GM-CSF.

10. The method of claim 8, wherein said polypeptide antigen is the tissue-specific tumor antigen prostatic acid phosphatase.

10 11. The method of claim 8, wherein the polypeptide complex is a fusion protein.

12. The method of claim 8, wherein said isolated antigen presenting cell is a dendritic cell.

13. A method of inducing a cytotoxic T-cell response in a vertebrate subject, comprising
15 contacting an isolated antigen presenting cell with a polypeptide complex comprising a dendritic cell-binding protein covalently linked to a polypeptide antigen selected from the group consisting of a tissue-specific tumor antigen and an oncogene product peptide antigen for a period of time effective to activate said antigen presenting cell, and
injecting said antigen presenting cell into the mammalian subject.

20

14. The method of claim 13, wherein said antigen presenting cell is a dendritic cell.

15. A polypeptide complex, comprising an isolated tissue-specific tumor antigen covalently linked to a dendritic cell-binding protein.

25

16. The polypeptide complex of claim 15, wherein said dendritic cell-binding protein is GM-CSF.

17. The polypeptide complex of claim 15, wherein said tissue-specific tumor antigen is
30 prostatic acid phosphatase.

18. The polypeptide complex of claim 15, wherein the complex is a fusion protein.

19. The polypeptide fusion protein complex of claim 15, which further includes, between said dendritic cell binding protein and said tissue-specific tumor antigen, a linker peptide.
20. A polypeptide complex, comprising an isolated oncogene product peptide antigen
5 covalently linked to a dendritic cell-binding protein.
21. The polypeptide complex of claim 20, wherein said dendritic cell-binding protein is GM-CSF.
- 10 22. The polypeptide complex of claim 20, wherein said oncogene product peptide antigen is selected from the group consisting of Her2, p21RAS, and p53.
23. The polypeptide complex of claim 20, wherein the complex is a fusion protein.
- 15 24. The polypeptide fusion protein of claim 20, which further includes, between said dendritic cell binding protein and said oncogene product antigen, a linker peptide.
25. An expression vector for producing an immunostimulatory fusion protein, comprising a nucleic acid molecule encoding a polypeptide complex consisting essentially of a dendritic cell-
20 binding protein and a polypeptide antigen selected from the group consisting of a tissue-specific tumor antigen, and an oncogene product peptide antigen, said nucleic acid molecule inserted into an expression vector, wherein said nucleic acid molecule is operably linked to a selected promoter able to initiate transcription in a selected host cell.
- 25 26. The expression vector of claim 25, wherein said dendritic cell-binding protein is GM-CSF and said polypeptide antigen is the tissue-specific tumor antigen prostatic acid phosphatase.
27. The expression vector of claim 25, wherein said dendritic cell-binding protein is GM-CSF and said polypeptide antigen is the oncogene product peptide antigen Her2.
- 30 28. A substantially purified nucleic acid molecule encoding a fusion protein consisting essentially of GM-CSF and prostatic acid phosphatase.

29. A substantially purified nucleic acid molecule encoding a fusion protein consisting essentially of GM-CSF and Her2.

30. An expression system for producing a fusion protein consisting essentially of a dendritic
5 cell-binding protein covalently linked to a polypeptide antigen selected from the group consisting of a tissue-specific tumor antigen and an oncogene product peptide antigen, comprising

a nucleic acid sequence encoding a dendritic cell-binding protein,

a nucleic acid sequence encoding the polypeptide antigen, each of said nucleic acid

10 sequences inserted into an expression vector, wherein said nucleic acid sequences are operably linked to a promoter able to initiate transcription in a selected host cell, and said expression vector is carried within the host cell.

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CGGCTCTCCT CAACATGAGA GCTGCACCCC TCCTCCTGGC CAGGGCAGCA 50
 MetArg AlaAlaProL euLeuLeuAl aArgAlaAla
 AGCCTTAGCC TTGGCTTCTT GTTCTGCTT TTTTCTGGC TAGACCGAAG 100
 SerLeuSerL euGlyPheLe uPheLeuLeu PhePheTrpL euAspArgSe
 TGTACTAGCC AAGGAGTTGA AGTTTGTGAC TTTGGTGTTC CGGCATGGAG 150
 rValLeuAla LysGluLeuL ysPheValTh rLeuValPhe ArgHisGlyA
 ACCGAAGTCC CATTGACACC TTTCCCACTG ACCCCATAAA GGAATCCTCA 200
 spArgSerPr oIleAspThr PheProThrA spProIleLy sGluSerSer
 TGGCCACAAG GATTTGGCCA ACTCACCCAG CTGGGCATGG AGCAGCATTa 250
 TrpProGlnG lyPheGlyGl nLeuThrGln LeuGlyMetG luGlnHisTy
 TGAACCTGGA GAGTATATAA GAAAGAGATA TAGAAAATTC TTGAATGAGT 300
 rGluLeuGly GluTyrIleA rgLysArgTy rArgLysPhe LeuAsnGluS
 CCTATAACA TGAACAGGTT TATATTCGAA GCACAGACGT TGAACCGACT 350
 erTyrLysHi sGluGlnVal TyrIleArgS erThrAspVa lAspArgThr
 TTGATGAGTG CTATGACAAA CCTGGCAGCC CTGTTTCCCC CAGAAGGTGT 400
 LeuMetSerA laMetThrAs nLeuAlaAla LeuPheProP roGluGlyVa
 CAGCATCTGG AATCCTATCC TACTCTGGCA GCCCATCCCG GTGCACACAG 450
 lSerIleTrp AsnProIleL euLeuTrpGl nProIlePro ValHisThrV
 TTCCTCTTTC TGAAGATCAG TTGCTATACC TGCCTTTCAG GAACTGCCCT 500
 alProLeuSe rGluAspGln LeuLeuTyrL euProPheAr gAsnCysPro
 CGTTTTCAAG AACTTGAGAG TGAGACTTTG AAATCAGAGG AATTCCAGAA 550
 ArgPheGlnG luLeuGluSe rGluThrLeu LysSerGluG luPheGlnLy
 GAGGCTGCAC CTTATAAGG ATTTTATAGC TACCTTGGGA AAATTTTCAG 600
 sArgLeuHis ProTyrLysA spPheIleAl aThrLeuGly LysLeuSerG
 GATTACATGG CCAGGACCTT TTTGGAATTT GGAGTAAAGT CTACGACCTT 650
 lyLeuHisGl yGlnAspLeu PheGlyIleT rpSerLysVa lTyrAspPro
 TTATATTGTG AGAGTGTTCa CAATTTCACT TTACCCTCCT GGGCCACTGA 700
 LeuTyrCysG luSerValHi sAsnPheThr LeuProSerT rpAlaThrGl
 GGACACCATG ACTAAGTTGA GAGAATTGTC AGAATTGTCC CTCCTGTCCC 750
 uAspThrMet ThrLysLeuA rgGluLeuSe rGluLeuSer LeuLeuSerL
 TCTATGGAAT TCACAAGCAG AAAGAGAAAT CTAGGCTCCA AGGGGTGTGTC 800
 euTyrGlyIl eHisLysGln LysGluLysS erArgLeuGl nGlyGlyVal
 CTGGTCAATG AAATCCTCAA TCACATGAAG AGAGCAACTC AGATACCAAG 850
 LeuValAsnG luIleLeuAs nHisMetLys ArgAlaThrG lnIleProSe
 CTACAAAAAA CTTATCATGT ATTCTGCGCA TGACACTACT GTGAGTGGCC 900
 rTyrLysLys LeuIleMetT yrSerAlaHi sAspThrThr ValSerGlyL
 TACAGATGGC GCTAGATGTT TACAACGGAC TCCTTCCTCC CTATGCTTCT 950
 euGlnMetAl aLeuAspVal TyrAsnGlyL euLeuProPr oTyrAlaSer
 TGCCACTTGA CGGAATTGTA CTTTGAGAAG GGGGAGTACT TTGTGGAGAT 1000
 CysHisLeuT hrGluLeuTy rPheGluLys GlyGluTyrP heValGlume
 GTACTATCGG AATGAGACGC AGCACGAGCC GTATCCCCTC ATGCTACCTG 1050
 tTyrTyrArg AsnGluThrG lnHisGluPr oTyrProLeu MetLeuProG

Fig. 1A

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GCTGCAGCCC TAGCTGTCCT CTGGAGAGGT TTGCTGAGCT GGTGGGCCCT	1100
lyCysSerPr oSerCysPro LeuGluArgP heAlaGluLe uValGlyPro	
GTGATCCCTC AAGACTGGTC CACGGAGTGT ATGACCACAA ACAGCCATCA	1150
ValIleProG lnAspTrpSe rThrGluCys MetThrThrA snSerHisGl	
AGGTACTGAG GACAGTACAG A <u>TTGGATC</u> CGC ACCCGCCCCG TCGCCCAGCC	1200
nGlyThrGlu AspSerThrA sp GlySerAl aProAlaArg SerProSerP	
CCAGCACACA GCCCTGGGAG CATGTGAATG CCATCCAGGA GGCCCGGCGT	1250
roSerThrGl nProTrpGlu HisValAsnA laIleGlnGl uAlaArgArg	
CTCCTGAACC TGAGTAGAGA CACTGCTGCT GAGATGAATG AAACAGTAGA	1300
LeuLeuAsnL euSerArgAs pThrAlaAla GluMetAsnG luThrValGl	
AGTCATCTCA GAAATGTTTG ACCTCCAGGA GCCGACCTGC CTACAGACCC	1350
uValIleSer GluMetPheA spLeuGlnGl uProThrCys LeuGlnThrA	
GCCTGGAGCT GTACAAGCAG GGCCTGCGGG GCAGCCTCAC CAAGCTCAAG	1400
rgLeuGluLe uTyrLysGln GlyLeuArgG lySerLeuTh rLysLeuLys	
GGCCCCCTGA CCATGATGGC CAGCCACTAC AAACAGCACT GCCCTCCAAC	1450
GlyProLeuT hrMetMetAl aSerHisTyr LysGlnHisC ysProProTh	
CCCGGAAACT TCCTGTGCAA CCCAGATTAT CACCTTTGAA AGTTTCAAAG	1500
rProGluThr SerCysAlaT hrGlnIleIl eThrPheGlu SerPheLysG	
AGAACCTGAA GGACTTTCTG CTTGTCATCC CCTTTGACTG CTGGGAGCCA	1550
luAsnLeuLy sAspPheLeu LeuValIleP roPheAspCy sTrpGluPro	
GTCCAGGAGT GAGACCGGCC AGATGAGGCT GGCCAAGC	1588
ValGlnGlu. . .	

Fig. 1B

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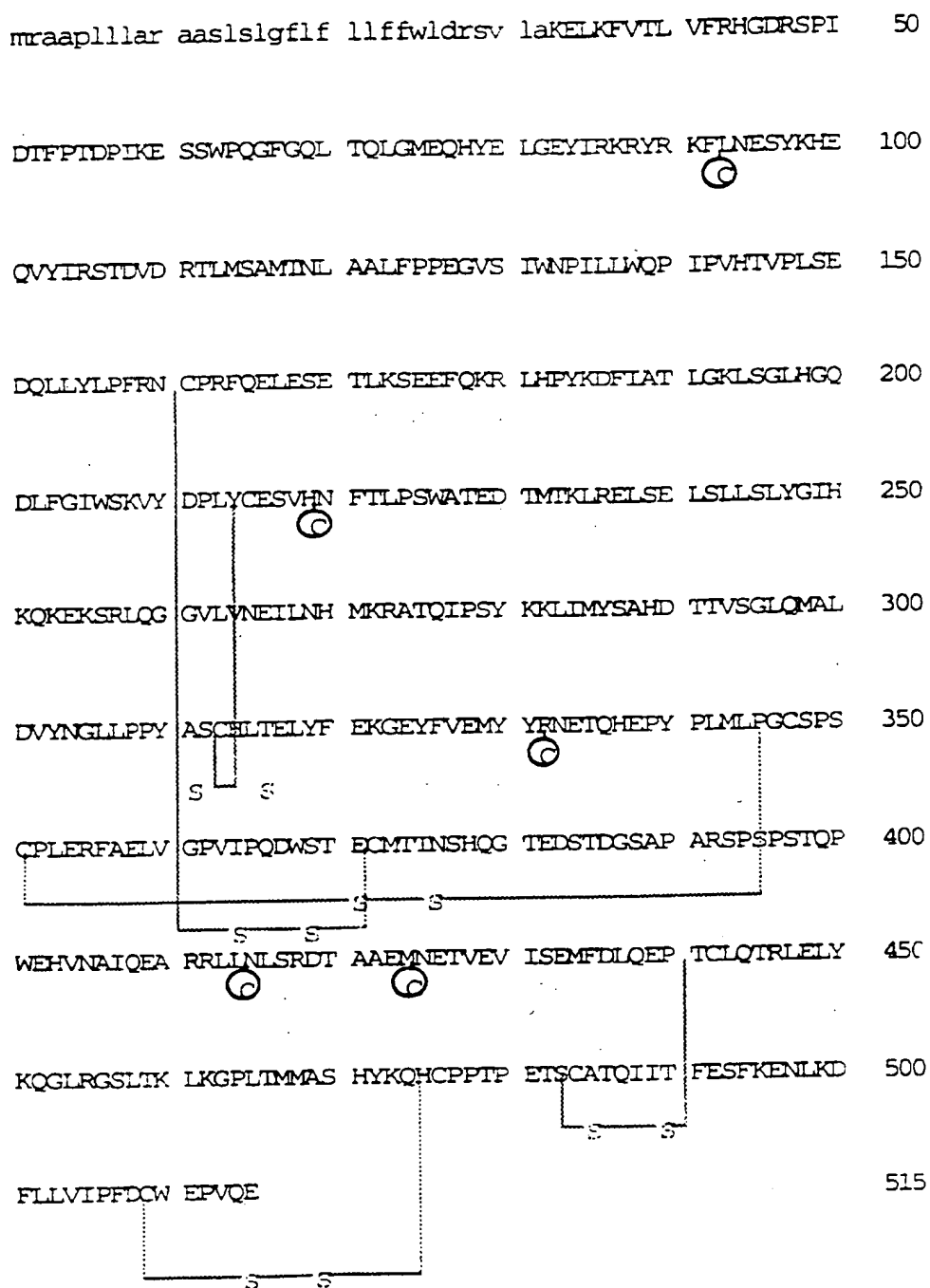


Fig. 2

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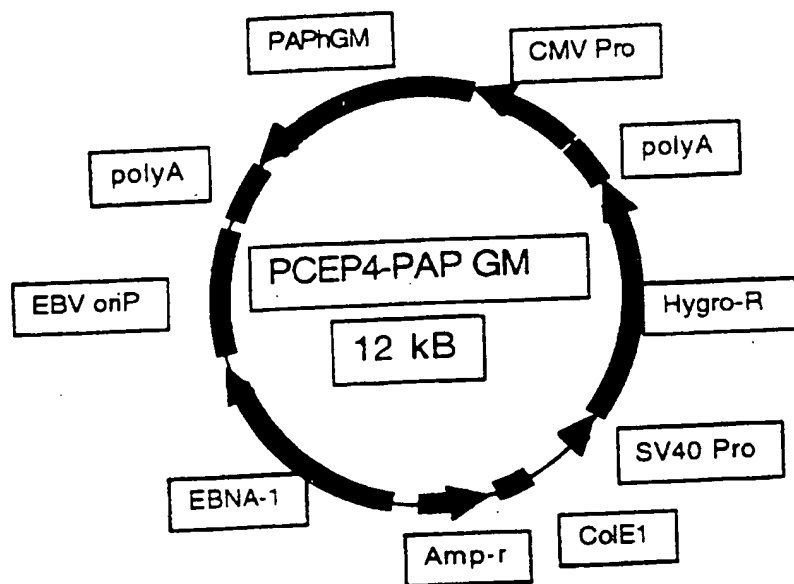


Fig. 3A

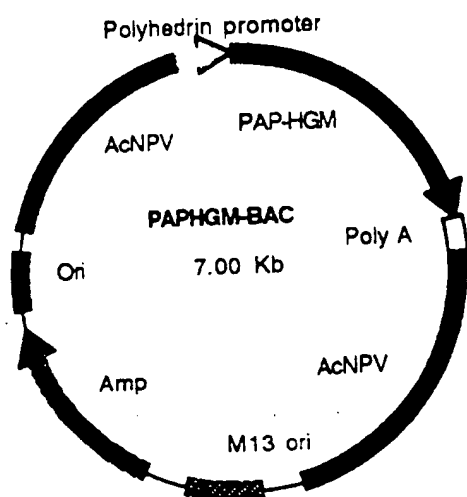


Fig. 3B

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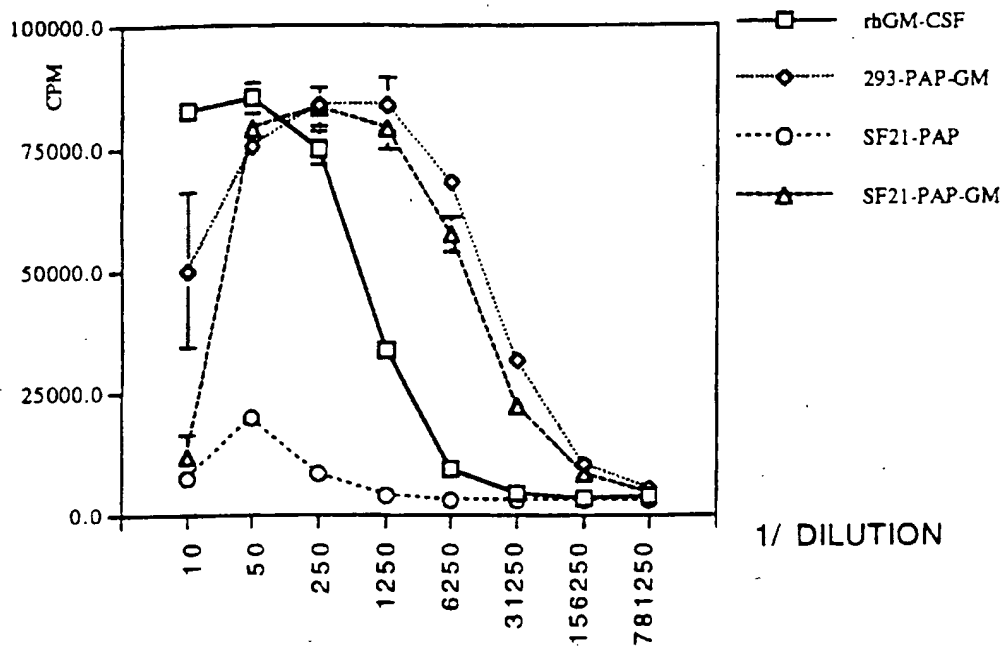


Fig. 4

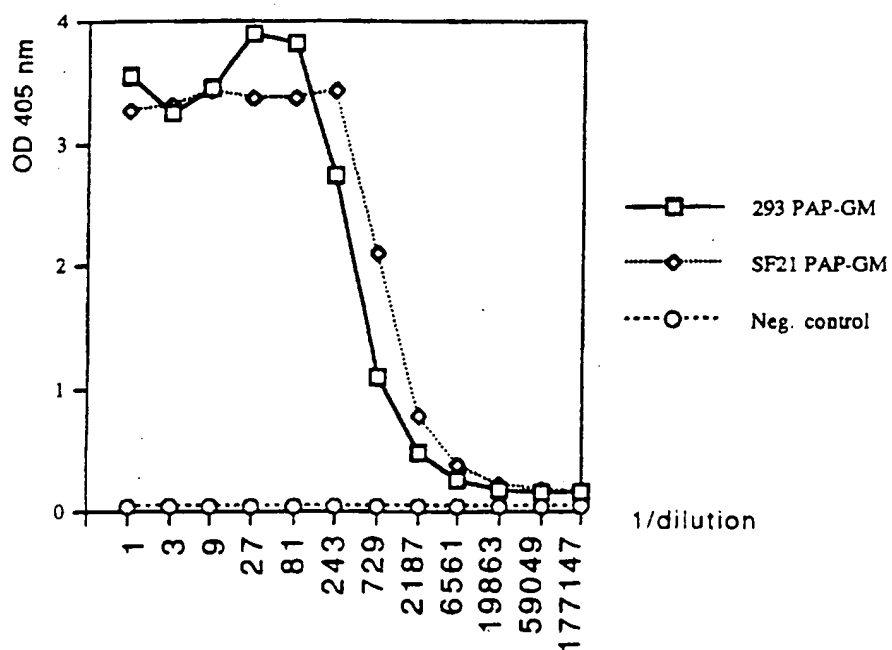


Fig. 5

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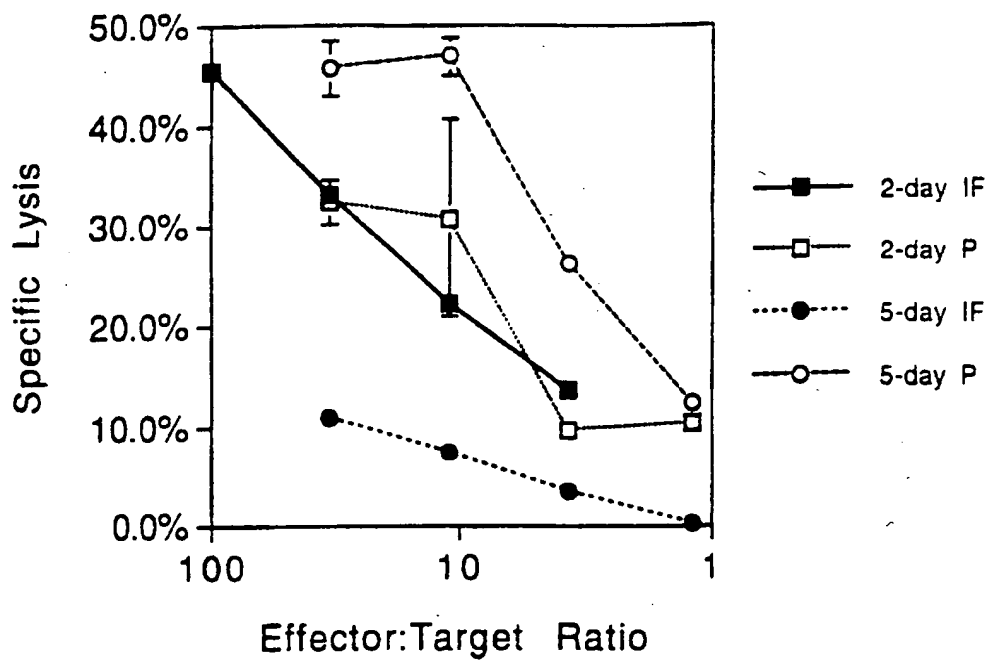


Fig. 6

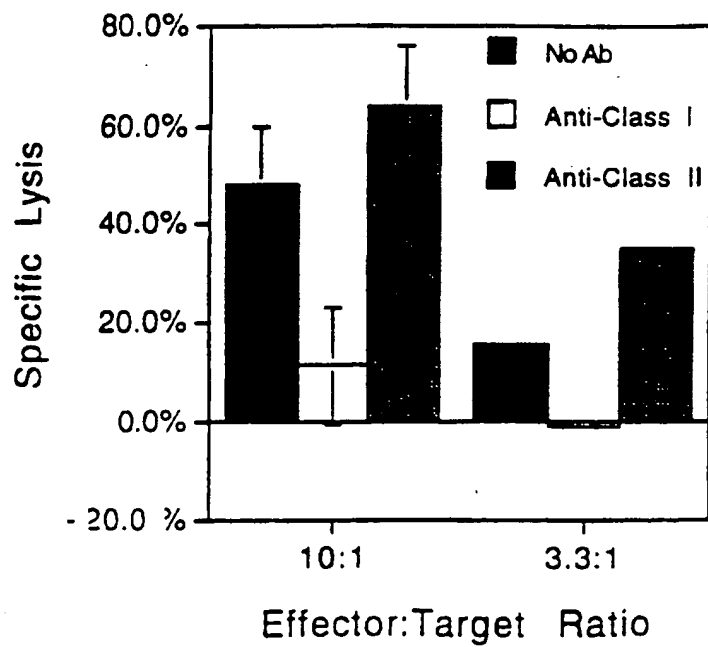


Fig. 7

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AGTGAGCACC	ATGGAGCTGG	CGGCCTGTG	CCGCTGGGGG	CTCCTCCTCG	CCCTCTTGCC	CCCCGGAGCC	GCGAG	75
MetGluLeuA	laAlaLeuCy	sArgTrpGly	LeuLeuLeuA	laLeuLeuPr	oProGlyAla	AlaSe		
CACCCAGTG	TGCACCGGCA	CAGACATGAA	GCTGCGGCTC	CCTGCCAGTC	CCGAGACCCA	CCTGGACATG	CTCCG	150
rThrGlnVal	CysThrGlyT	hrAspMetLy	sLeuArgLeu	ProAlaSerP	roGluThrHi	sLeuAspMet	LeuAr	
CCACCTCTAC	CAGGCGTGCC	AGGTGGTGCA	GGGAAACCTG	GAATCACCCT	ACCTGCCCAC	CAATGCCAGC	CTGTC	225
gHisLeuTyr	GlnGlyCysG	lnValValGl	nGlyAsnLeu	GluLeuThrT	yrLeuProTh	rAsnAlaSer	LeuSe	
CTTCCTGCAG	GATATCCAGG	AGGTGCAGGG	CTACGTGCTC	ATCGCTCACA	ACCAAGTGAG	GCAGGTCCCA	CTGCA	300
rPheLeuGln	AspIleGlnG	luValGlnGl	yTyrValLeu	IleAlaHisA	snGlnValAr	gGlnValPro	LeuGl	
GAGGCTGCGG	ATTGTGCGAG	GCACCCAGCT	CTTTGAGGAC	AACCTATGCCC	TGGCCGTGCT	AGACAATGGA	GACCC	375
nArgLeuArg	IleValArgG	lyThrGlnLe	uPheGluAsp	AsnTyrAlaL	euAlaValLe	uAspAsnGly	AspPr	
GCTGAACAAT	ACCACCCCTG	TCACAGGGGC	CTCCCCAGGA	GGCCTGCGGG	AGCTGCAGCT	TCGAAGCCTC	ACAGA	450
oLeuAsnAsn	ThrThrProV	alThrGlyAl	aSerProGly	GlyLeuArgG	luLeuGlnLe	uArgSerLeu	ThrGl	
GATCTTGAAA	GGAGGGGTCT	TGATCCAGCG	GAACCCCCAG	CTCTGCTACC	AGGACACGAT	TTTGTGGAAG	GACAT	525
uIleLeuLys	GlyGlyValL	euIleGlnAr	gAsnProGln	LeuCysTyrG	lnAspThrIl	eLeuTrpLys	AspIl	
CTTCACAAG	AACAACCAGC	TGGCTCTCAC	ACTGATAGAC	ACCAACCGCT	CTCGGGCCTG	CCACCCCTGT	TCCTC	600
ePheHisLys	AsnAsnGlnL	euAlaLeuTh	rLeuIleAsp	ThrAsnArgS	erArgAlaCy	sHisProCys	SerPr	
GATGTGTAAG	GGCTCCCGCT	GCTGGGGAGA	GAGTTCTGAG	GATTGTGAGA	GCCTGACGCG	CACTGTCTGT	GCCGG	675
oMetCysLys	GlySerArgC	ysTrpGlyGl	uSerSerGlu	AspCysGlnS	erLeuThrAr	gThrValCys	AlaGl	
TGGCTGTGCC	CGCTGCAAGG	GGCCACTGCC	CACTGACTGC	TGCCATGAGC	AGTGTGCTGC	CGGCTGCACG	GGCCC	750
yGlyCysAla	ArgCysLysG	lyProLeuPr	oThrAspCys	CysHisGluG	lnCysAlaAl	aGlyCysThr	GlyPr	
CAAGCACTCT	GACTGCCTGG	CCTGCCTCCA	CTTCAACCAC	AGTGGCATCT	GTGAGCTGCA	CTGCCCAGCC	CTGCT	825
oLysHisSer	AspCysLeuA	laCysLeuHi	sPheAsnHis	SerGlyIleC	ysGluLeuHi	sCysProAla	LeuVa	
CACCTACAAC	ACAGACACGT	TTGAGTCCAT	GCCCAATCCC	GAGGGCCCGT	ATACATTCGG	CGCCAGCTGT	GTGAC	900
lThrTyrAsn	ThrAspThrP	heGluSerMe	tProAsnPro	GluGlyArgT	yrThrPheGl	yAlaSerCys	ValTh	
TGCTGTCCC	TACAACCTACC	TTTCTACGGA	CGTGGGATCC	TGCACCCCTC	TCTGCCCCCT	GCACAACCAA	GAGGT	975
rAlaCysPro	TyrAsnTyrL	euSerThrAs	pValGlySer	CysThrLeuV	alCysProLe	uHisAsnGln	GluVa	
GACAGCAGAG	GATGGAACAC	AGCGGTGTGA	GAAGTGACGC	AAGCCCTGTG	CCCGAGTGTG	CTATGGTCTG	GGCAT	1050
lThrAlaGlu	AspGlyThrG	lnArgCysGl	uLysCysSer	LysProCysA	laArgValCy	sTyrGlyLeu	GlyMe	
GGAGCACTTG	CGAGAGGTGA	GGGCAGTTAC	CAGTGCCAAT	ATCCAGGAGT	TTGCTGGCTG	CAAGAAGATC	TTTGG	1125
tGluHisLeu	ArgGluValA	rgAlaValTh	rSerAlaAsn	IleGlnGluP	heAlaGlyCy	sLysLysIle	PheGl	
GAGCCTGGCA	TTTCTGCCGG	AGAGCTTTGA	TGGGGACCCA	GCCTCCAACA	CTGCCCCGCT	CCAGCCAGAG	CAGCT	1200
ySerLeuAla	PheLeuProG	luSerPheAs	pGlyAspPro	AlaSerAsnT	hrAlaProLe	uGlnProGlu	GlnLe	
CCAAGTGTIT	GAGACTCTGG	AAGAGATCAC	AGGTTACCTA	TACATCTCAG	CATGGCCGGA	CAGCCTGCCT	GACCT	1275
uGlnValPhe	GluThrLeuG	luGluIleTh	rGlyTyrLeu	TyrIleSerA	laTrpProAs	pSerLeuPro	AspLe	
CAGCGTCTTC	CAGAACCTGC	AAGTAATCCG	GGGACGAATT	CTGCACAATG	GCGCTTACTC	GCTGACCCCTG	CAAGG	1350
uSerValPhe	GlnAsnLeuG	lnValIleAr	gGlyArgIle	LeuHisAsnG	lyAlaTyrSe	rLeuThrLeu	GlnGl	
GCTGGGCATC	AGCTGGCTGG	GGCTGCGCTC	ACTGAGGGAA	CTGGGCAGTG	GACTGGCCCT	CATCCACCAT	AACAC	1425
yLeuGlyIle	SerTrpLeuG	lyLeuArgSe	rLeuArgGlu	LeuGlySerG	lyLeuAlaLe	uIleHisHis	AsnTh	
CCACCTCTGC	TTGCTGCACA	CGGTGCCCTG	GGACCAGCTC	TTTCGGAACC	CGCACCAAGC	TCTGCTCCAC	ACTGC	1500
rHisLeuCys	PheValHisT	hrValProTr	pAspGlnLeu	PheArgAsnP	roHisGlnAl	aLeuLeuHis	ThrAl	
CAACCGGCCA	GAGGACGAGT	GTGTGGGCGA	GGCCCTGGCC	TGCCACCAGC	TGTGCGCCCG	AGGGCACTGC	TGGGG	1575
aAsnArgPro	GluAspGluC	ysValGlyGl	uGlyLeuAla	CysHisGlnL	euCysAlaAr	gGlyHisCys	TrpGl	
TCCAGGGCCC	ACCCAGTGTG	TCAACTGCAG	CCAGTTCCCT	CGGGGCCAGG	AGTGCGTGA	GGAATGCCGA	GTACT	1650
yProGlyPro	ThrGlnCysV	alAsnCysSe	rGlnPheLeu	ArgGlyGlnG	luCysValGl	uGluCysArg	ValLe	
GCAGGGGCTC	CCCAGGGAGT	ATGTGAATGC	CAGGCACGTG	TGCGCGTGCC	ACCCTGAGTG	TCAGCCCCAG	AATGG	1725
uGlnGlyLeu	ProArgGluT	yrValAsnAl	aArgHisCys	LeuProCysH	isProGluCy	sGlnProGln	AsnGl	
CTCAGTGACC	TGTTTTGGAC	CGGAGGCTGA	CCAGTGTGTG	GCCTGTGCCC	ACTATAAGGA	CCCTCCCTTC	TGCGT	1800
ySerValThr	CysPheGlyP	roGluAlaAs	pGlnCysVal	AlaCysAlaH	isTyrLysAs	pProProPhe	CysVa	

Fig. 8A

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GGCCCGCTGC CCCAGCGGTG TGAAACCTGA CCTCTCCTAC ATGCCCATCT GGAAGTTTCC AGATGAGGAG GGCGC 1875
lAlaArgCys ProSerGlyV allLysProAs pLeuSerTyr MetProileT rpLysPhePr oAspGluGlu GlyAl
ATGCCAGCCT TGCCCCATCA ACTGCACCCA CTCCTGTGTG GACCTGGATG ACAAGGGCTG CCCC GCCGAG CAGAG 1950
aCysGlnPro CysProileA snCysThrHi sSerCysVal AspLeuAspa spLysGlyCy sProAlaGlu GlnAr
AGCCAGCCCT CTGACGTCC TCGAGGCACC CGCCCGCTCG CCCAGCCCCA GCACACAGCC CTGGGAGCAT GTGAA 2025
gAlaSerPro LeuThrSerL euGluAlaPr oAlaArgSer ProSerProS erThrGlnPr oTrpGluHis ValAs
TGCCATCCAG GAGGCCCGGC GTCTCCTGAA CCTGAGTAGA GACACTGCTG CTGAGATGAA TGAAACAGTA GAAGT 2100
nAlaIleGln GluAlaArgA rgLeuLeuAs nLeuSerArg AspThrAlaa laGluMetAs nGluThrVal GluVa
CATCTCAGAA ATGTTTGACC TCCAGGAGCC GACCTGCCTA CAGACCCGCC TGGAGCTGTA CAAGCAGGCC CTGCG 2175
lIleSerGlu MetPheAspL euGlnGluPr oThrCysLeu GlnThrArgL euGluLeuTy rLysGlnGly LeuAr
GGGCAGCCTC ACCAAGCTCA AGGGCCCTT GACCATGATG GCCAGCCACT ACAAACAGCA CTGCCCTCCA ACCCC 2250
gGlySerLeu ThrLysLeuL ysGlyProLe uThrMetMet AlaSerHist yrLysGlnHi sCysProPro ThrPr
GGAAACTTCC TGTGCAACCC AGATTATCAC CTTTGAAAGT TTCAAAGAGA ACCTGAAGGA CTTTCTGCTT GTCAT 2325
oGluThrSer CysAlaThrG lnIleIleTh rPheGluSer PheLysGluA snLeuLysAs pPheLeuLeu ValIl
CCCCTTTGAC TGCTGGGAGC CAGTCCAGGA GTGAGACCGG CCAGATGAGG CTGGCCAAGC 2385
eProPheAsp CysTrpGluP roValGlnGl u....

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Fig. 8B

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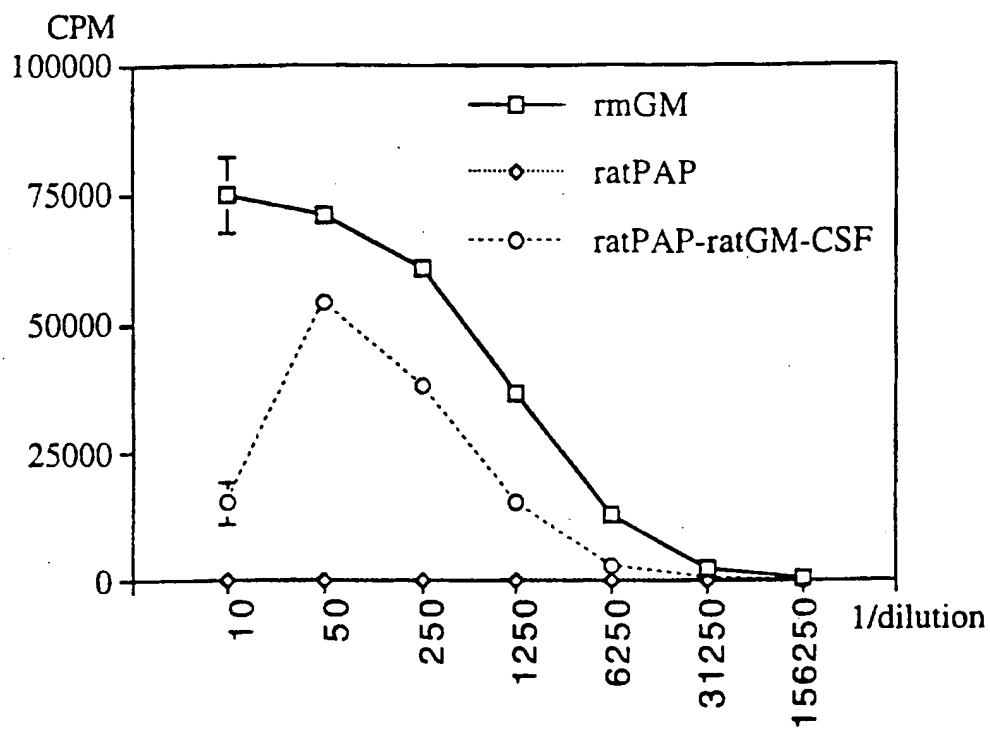


Fig. 9

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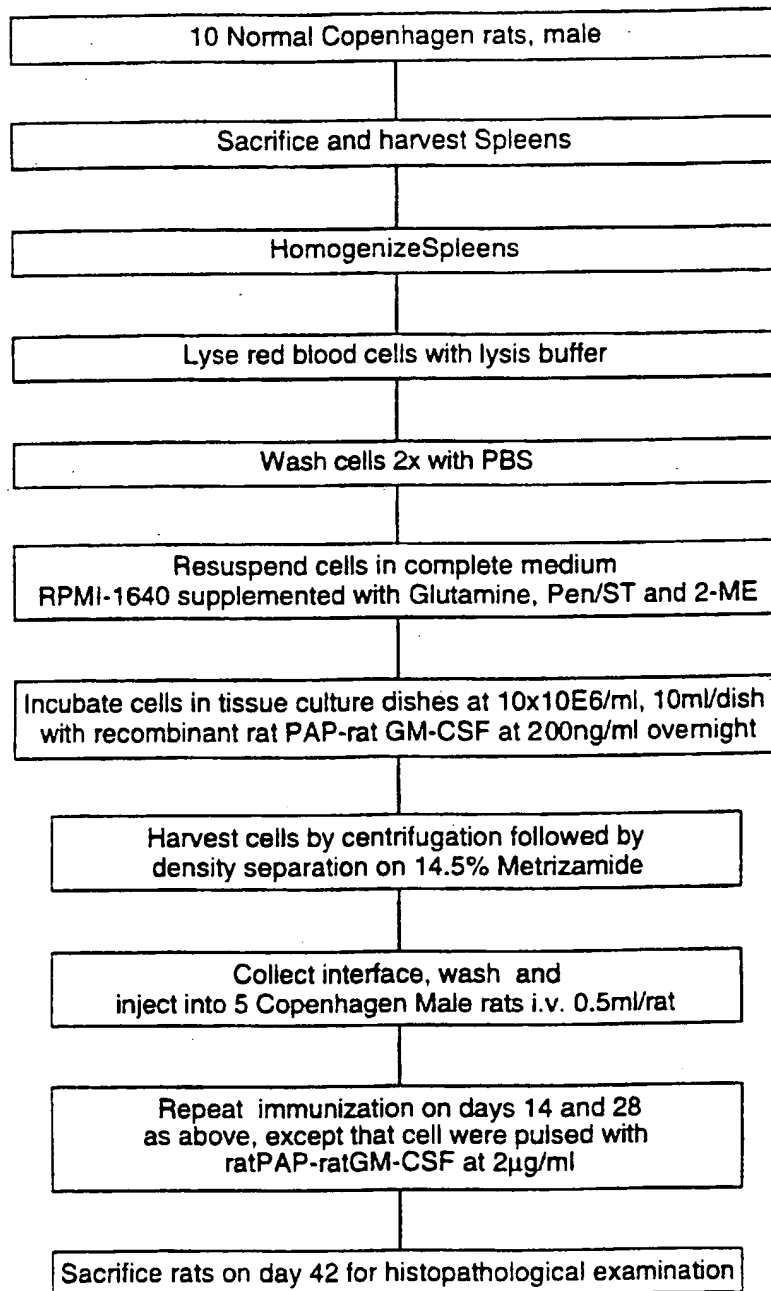


Fig. 10

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TCACCTGCCAT	GGAGGAGCCG	CAGTCAGATC	CTAGCGTCCA	GGCCCCCTCTG	AGTCAGGAAA	60
Me tGluGluPro	GlnSerAspP	roSerValGl	uProProLeu	SerGlnGluT		
CATTTTCAGA	CCTATGGAAA	CTACTTCCTG	AAAACAACGT	TCTGTCCCCC	TTGCCGTCCC	120
hrPheSerAs	pLeuTrpLys	LeuLeuProG	luAsnAsnVa	lLeuSerPro	LeuProSerG	
AAGCAATGGA	TGATTTGATG	CTGTCCCCGG	ACCATATTGA	ACAATGGTTC	ACTGAAGACC	180
lnAlaMetAs	pAspLeuMet	LeuSerProA	spAspIleGl	uGlnTrpPhe	ThrGluAspP	
CAGGTCCAGA	TGAAGCTCCC	AGAATGCCAG	AGGCTGCTCC	CCCCGTGGCC	CCTGCACCAG	240
roGlyProAs	pGluAlaPro	ArgMetProG	luAlaAlaPr	oProValAla	ProAlaProA	
CAGCTCCTAC	ACCGGGGGCC	CCTGCACCAG	CCCCCTCCTG	GGCCCTGTCA	TCTTCTGTCC	300
laAlaProTh	rProAlaAla	ProAlaProA	laProSerTr	pProLeuSer	SerSerValP	
CTTCCCAGAA	AACCTACCAG	GGCAGCTACG	GTTCCTCTCT	GGGCTTCTTG	CATTCTGGGA	360
roSerGlnLy	sProValGln	GlySerTyrG	lyPheArgLe	uGlyPheLeu	HisSerGlyT	
CAGCCAAGTC	TGTGACTTGC	ACGTAATCCC	CTGCCCTCAA	CAAGATGTTT	TGCCAACTGG	420
hrAlaLysSe	rValThrCys	ThrTyrSerP	roAlaLeuAs	nLysMetPhe	CysGlnLeuA	
CCAAGACCTG	CCCTGTGCAG	CTGTGGGTTC	ATTCCACACC	CCCGCCCGGC	ACCCCGCTCC	480
laLysThrCy	sProValGln	LeuTrpValA	spSerThrPr	oProProGly	ThrArgValA	
GCGCCATGCG	CATCTACAAG	CAGTCACAGC	ACATGACGGA	GGTGTGTAGG	CGCTGCCCCC	540
rgAlaMetAl	aIleTyrLys	GlnSerGlnH	isMetThrGl	uValValArg	ArgCysProH	
ACCATGAGCG	CTGCTCAGAT	AGCGATGGTC	TGGCCCCCTCC	TCAGCATCTT	ATCCGAGTGG	600
isHisGluAr	cCysSerAsp	SerAspGlyL	euAlaProPr	oGlnHisLeu	IleArgValG	
AAGGAAATTT	GCGTGTGGAG	TATTTGGATG	ACAGAAACAC	TTTTCGACAT	AGTGTGGTGG	660
luGlyAsnLe	uArgValGlu	TyrLeuAspA	spArgAsnTh	rPheArgHis	SerValValV	
TGCCCTATGA	GGCGCTGAG	GTGTGGCTCTG	ACTGTACCAC	CATCCACTAC	AACTACATGT	720
alProTyrGl	uProProGlu	ValGlySerA	spCysThrTh	rIleHisTyr	AsnTyrMetC	
GTAACAGTTC	CTGCATGGGC	GGCATGAACC	GGAGGCCCAT	CCTCACCATC	ATCACACTGG	780
ysAsnSerSe	rCysMetGly	GlyMetAsnA	rgArgProIl	eLeuThrIle	IleIthrLeuG	
AAGACTCCAG	TGGTAATCTA	CTGGGACGGA	ACAGCTTTGA	GGTGGGTGTT	TGTGCCCTGTC	840
luAspSerSe	rGlyAsnLeu	LeuGlyArgA	snSerPheGl	uValArgVal	CysAlaCysP	
CTGGGAGAGA	CCGGCGCACA	GAGGAAGAGA	ATCTCCGCAA	GAAAGGGGAG	CCTCACCACG	900
roGlyArgAs	pArgArgThr	GluGluGluA	snLeuArgLy	sLysGlyGlu	ProHisHisG	
AGCTGCCCCC	AGGGAGCACT	AAGCGAGCAC	TGCCCAACAA	CACCAGCTCC	TCTCCCCAGC	960
luLeuProPr	oGlySerThr	LysArgAlaL	tuProAsnAs	nThrSerSer	SerProGlnP	
CAAAGAAGAA	ACCACTGGAT	GGAGAATATT	TCACCCCTCA	GATCCGTGGG	CGTGAGCGCT	1020
roLysLysLy	sProLeuAsp	GlyGluTyrP	heThrLeuGl	nIleArgGly	ArgGluArgP	
TCGAGATGTT	CCGAGAGCTG	AATGAGGCCT	TGGAAGTCAA	GGATGCCCAG	GCTGGGAAGG	1080
heGluMetPh	eArgGluLeu	AsnGluAlaL	euGluLeuLy	sAspAlaGln	AlaGlyLysG	
AGCCAGGGGG	GAGCAGGGCT	CACTCCAGCC	ACCTGAAGTC	CAAAAAGGGT	CAGTCTACCT	1140
luProGlyGl	ySerArgAla	HisSerSerH	isLeuLysSe	rLysLysGly	GlnSerThrs	
CCCGCCATAA	AAAATCATG	TTCAAGACAG	AAGGGCCTGA	CTCAGACTCT	AGCTCCGCAC	1200
erArgHisLy	sLysLeuMet	PheLysThrG	luGlyProAs	pSerAspSer	ArgSerAlaP	
CCGCCCGCTC	GGCCAGCCCC	AGCACACAGC	CCTGGGAGCA	TGTGAATGCC	ATCCAGGAGG	1260
roAlaArgSe	rProSerPro	SerThrGlnP	roTrpGluHi	sValAsnAla	IleGlnGluA	
CCCGGGCTCT	CCTGAACCTG	AGTAGAGACA	CTGCTGCTGA	GATGAATGAA	ACAGTAGAAG	1320
laArgArgLe	uLeuAsnLeu	SerArgAspT	hrAlaAlaGl	uMetAsnGlu	ThrValGluV	
TCATCTCAGA	AATGTTTGAC	CTCCAGGAGC	CGAAGCTGCT	ACAGACCCGC	CTGGAGCTGT	1380
alIleSerGl	uMetPheAsp	LeuGlnGluP	roThrCysLe	uGlnThrArg	LeuGluLeuT	
ACAAGCAGGG	CCTGCCGGGC	AGCCTCACCA	AGCTCAAGGG	CCCTTGACCC	ATGATGGCCA	1440
yrLysGlnGl	yLeuArgGly	SerLeuThrL	ysLeuLysGl	yProLeuThr	MetMetAlas	
GCCACTACAA	ACAGCACTGC	CCTCCAACCC	CGGAACTTTC	CTGTGCAACC	CAGATTATCA	1500
erHisTyrLy	sGlnHisCys	ProProThrP	roGluThrSe	rCysAlaThr	GlnIleIleT	
CCTTTGAAAG	TTTCAAGAG	AACCTGAAGG	ACTTTCTGCT	TGTCATCCCC	TTTGACTGCT	1560
hrPheGluSe	rPheLysGlu	AsnLeuLysA	spPheLeuLe	uValIlePro	PheAspCysT	
GGGAGCCAGT	CCAGGAGATG	AGAGGATGCG	ATCAACATCA	CCATCACTGA		1610
rpGluProVa	lGlnGluMet	ArgGlySerH	isHisHisHi	sHisHis...		

Fig. 11

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MEEPQSDPSV	EPPLSQETFS	DLWKLLPENN	VLSPPLPSQAM	DDLMLSPDDI	50
EQWFTEDPGP	DEAPRMPEAA	PPVAPAPAAP	TPAAPAPAPS	WPLSSSVPSQ	100
KTYQGSYGFR	LGFLHSGTAK	SVTCTYSPAL	NKMFCQLAKT	CPVQLWVDST	150
PPPGTRVRAM	AIYKQSQHMT	EVVRRCPHHE	RCSDSDGLAP	POHLIRVEGN	200
LRVEYLDDRN	TFRHSVVVPY	EPPEVGSDCT	TIHYNMCNS	SCMGGMNRRP	250
ILTIITLED	SGNLLGRNSF	EVRVCACPGR	DRRTEENLR	KKGEPHHELP	300
PGSTKRALPN	NTSSSPQPKK	KPLDGEYFTL	QIRGRERFEM	FRELNEALEL	350
KDAQAGKEPG	GSRAHSSHLK	SKKGQSTSRH	KKLMFKTEGP	DSD <u>SR</u> SAPAR	400
SPSPSTQPWE	HVNAIQEARR	LLNLSRDTAA	EMNETVEVIS	EMFDLQEPTC	450
LQTRLELYKQ	GLRGS�TKLK	GPLTMMASHY	KQHCPTPET	SCATQIITFE	500
SFKENLKDFL	LVIPFDCWEP	VQEMRGSHHH	HHH		533

Fig. 12

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/20241

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N15/27 C12N15/55 C12N15/62 C12N9/16
C12N5/08 C07K14/535 C07K14/47 C07K14/71 A61K35/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATURE, vol. 344, 26 April 1990, pages 873-875, XP002031410 TAKAHASHI, H., ET AL.: "INDUCTION OF CD8+ CYTOTOXIC T CELLS BY IMMUNIZATION WITH PURIFIED HIV-1 ENVELOPE PROTEIN IN ISCOMs" see the whole document ---	1
X	WO 95 23814 A (NAT JEWISH CENTER FOR IMMUNOLO ; KAPPLER JOHN W (US); MARRACK PHILI) 8 September 1995 see the whole document ---	1
X	WO 94 21287 A (US HEALTH) 29 September 1994 see the whole document ---	1,2
-/--		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *A* document member of the same patent family

Date of the actual completion of the international search

27 May 1997

Date of mailing of the international search report

03.06.97

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Holtorf, S

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/20241

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATURE, vol. 362, 22 April 1993, pages 755-758, XP002031411 TAO, M.-H., ET AL.: "IDIOTYPE/GRANULOCYTE - MACROPHAGE COLONY-STIMULATION FACTOR FUSION PROTEIN AS A VACCINE FOR B-CELL LYMPHOMA" see the whole document ---	15,16, 18,25,30
P,X	WO 96 37107 A (SCRIPPS RESEARCH INST) 28 November 1996 see the whole document ---	1
P,X	VACCINE, vol. 14, no. 3, 1996, pages 230-236, XP002031412 TARPEY, I., ET AL.: "PRIMING IN VIVO AND QUANTIFICATION IN VITRO OF CLASS I MHC-RESTRICTED CYTOTOXIC T CELLS TO HUMAN PAPILLOMA VIRUS TYPE 11 EARLY PROTEINS (E6 AND E7) USING IMMUNOSTIMULATING COMPLEXES (ISCOMs)" see the whole document ---	1
P,X	WO 96 22377 A (ARMITAGE IAN M ;SANDLIE INGER (NO); BOGEN BJARNE (NO)) 25 July 1996 see the whole document ---	1,2,7,8, 11,12, 15,18, 20,22, 23,25,30
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 89, September 1992, pages 7871-7875, XP002031413 STAUSS, H.J., ET AL.: "INDUCTION OF CYTOTOXIC T LYMPHOCYTES WITH PEPTIDES IN VITRO: IDENTIFICATION OF CANDIDATE T-CELL EPITOPES IN HUMAN PAPILLOMA VIRUS" cited in the application see the whole document ---	1-30
A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 160, no. 1, 1989, pages 79-86, XP002031414 SHARIEF, F.S., ET AL.: "HUMAN PROSTATE ACID PHOSPHATASE: cDNA CLONING, GENE MAPPING, AND PROTEIN SEQUENCE HOMOLOGY WITH LYSOSOMAL ACID PHOSPHATASE" see the whole document ---	1-30
5 1	A WO 95 21862 A (BRIGHAM & WOMENS HOSPITAL) 17 August 1995 see the whole document -----	1-30

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/20241

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 13, 14
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 13, 14 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/US 96/20241

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9523814 A	08-09-95	AU 2094295 A CA 2184810 A	18-09-95 05-09-95
WO 9421287 A	29-09-94	AU 6363094 A CA 2158281 A EP 0692973 A	11-10-94 29-09-94 24-01-96
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WO 9622377 A	25-07-96	AU 4453896 A	07-08-96
WO 9521862 A	17-08-95	US 5550214 A AU 1914595 A	27-08-96 29-08-95